LIPOPROTEIN LOCALIZATION SIGNALS AND EVALUATION OF NOVEL DRUG TARGETS TO COMBAT TUBERCULOSIS

Dissertation
zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich

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Zürich 2011

To my parents

Ich erlaube mir die Bemerkung, dass mein wichtigstes Arbeitsinstrument nicht der Computer, sondern der Papierkorb ist.

(Hans Vontobel)

Acknowledgements

I would like to express my heartfelt gratitude to the many people who made this thesis possible.

First and foremost I would like to thank my supervisors Prof. Dr. Erik C. Böttger and Prof. Dr. Peter Sander. I am grateful for the opportunity to work in their laboratory, their inspiration and their support for my work. Peter, our lively discussions and your faithful guidance during this thesis were very important to me. Also I would like to thank Prof. Dr. Leo Eberl from the Department of Microbiology (UZH) for joining my thesis committee.

The close and straightforward collaboration with Marc Gitzinger and Dr. Marcel Tigges from the Department of Biosystems Science and Engineering (ETHZ) and Prof. Dr. Wilfried Weber from the Centre for Biological Signalling Studies (Albert-Ludwigs-University Freiburg) created the fundament for a decisive part of this work. Further, I would like to thank Prof. Dr. Menico Rizzi from the DiSCAFF in Novara and Dr. György Kéri from VICHEM, Hungary for providing me with NAD-Pathway inhibitors.

Claudia Ritter, Ute Friedel and Rico Hömke from the Mycobacteriology unit at the IMM assisted me with the development of drug screening assays with the MGIT-system.

Thanks also to my Master students Bea Wochele and Juliane Brülle for entrusting me with their tutoring.

I would further like to thank the present and former members of the research groups of Peter, Burkhard and Erik: Mandana, Sonja, Roger, Martin, Doro, Michael, Caro, Silvana, Christian, Sven, Tanja, Sarath, Akshay, Andreas, Agnese, Reto, Petra, Peter, Beat, Tobi, Michael, Mihai, Rashid, Dimitri, Tanja, Martin and Stefan. You all contributed to a welcoming atmosphere at the institute.

Special thanks to the diagnostic ladies' crew Silke, Silke and Diana, who lightened up my day.

This work would not have been possible without the enlivening inputs and distractions of my closest friends Be, Chantal, Chrigi, Fi, Mitja and Vivi. Your patience, encouragement and exhilaration provided me with enormous energy.

Last but not least I want to thank my parents and my sister for their unswerving support throughout my studies.

Thank you.

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Summary

With more than 9 million new cases and nearly 2 million deaths per year, Tuberculosis still remains a major global burden. The causative agent of the disease, *Mycobacterium tuberculosis*, belongs to the group of slow growing, GC-rich Gram-positive bacteria. Mycobacteria in general are characterized by a unique cell envelope, which forms a barrier for uptake of numerous chemical compounds. Therefore, treatment of Tuberculosis is naturally lenghty and complex. Recently an increase in drug resistant or multidrug-resistant (MDR) Tuberculosis has been observed. MDR is due to acquired mutations in several genes, conferring resistance to a wide spectrum of antibiotics. Even though *M. tuberculosis* has been described a century ago, the complete mechanisms of infection and virulence are not yet fully understood. Upon inhaling droplets containing *M. tuberculosis*, the bacterium is able to survive in lung alveolar macrophages and to persist in the body for decades. A class of secreted proteins, the so called lipoproteins, have been shown to be involved in several processes of these host-pathogen interactions. The aims of this study were 1) the characterization of the transport of lipoproteins to the cell wall of mycobacteria and 2) the evaluation of potential new drug targets to fight Tuberculosis including the enhancement of mycobacterial drugs used nowadays.

In order to investigate the transport of lipoproteins to the cell wall of mycobacteria, several lipoproteins of *M. tuberculosis* have been expressed in *Mycobacterium smegmatis*, a mycobacterial model organism. Using subcellular fractionation techniques, these reporter proteins were localized either in the cytoplasmic membrane or in the cell wall. Lipoproteins are defined by the so called lipobox at the N-terminus, which contains in its mature form a universally conserved and lipidated cysteine. In *Escherichia coli*, the very well characterized Gram-negative bacterium, lipoproteins are transported from the inner membrane to the outer membrane by the Lol-system, unless they contain a retention signal located at position +2 after the cysteine. As mycobacteria miss a transporter similar to the Lol-system, transport and signal sequences for transport of lipoproteins to the cell wall remain unclear. By site-directed mutagenesis, several hybrid lipoprotein reporter constructs have been generated, expressed in *M. smegmatis* and subjected to subcellular fractionation. The results of these experiments indicate that a region of 10 amino acids subsequent to the cysteine of the lipobox acts as a positive signal for the transport to the cell wall.

The standard treatment to fight Tuberculosis consits of a combination of several first-line drugs. With the rise of multidrug-resistant strains of *M. tuberculosis* (MDR-TB), second-line drugs have been brought back into focus. These antibiotics are second choice beause of either

a difficult mode of application (injectable drugs) or are more toxic and cause severe side-effects. One of those second-line drugs is ethionamide (ETH) which is a prodrug and thus has to be activated within the bacterium. The activation is performed by the enzyme EthA, which is naturally repressed by EthR. By screening of a chemical compound library generated by a synthetic mammalian gene circuit, the substance 2-phenylethyl-butyrate (2-PEB) was found to release EthR efficiently. This study shows by applying drug susceptibility testing, that the combination of 2-PEB and ETH has a synergistic effect on growth of mycobacteria, even on MDR-strains. Therefore we could show that ETH can be applied in sub-inhibitory concentrations against *M. tuberculosis*, which in turn could reduce the potential side-effects of this important second-line drug.

The last part of this work evaluates a new promising class of antibiotics, the NAD-synthetase inhibitors. NAD^+ is a coenzyme involved in redox reactions in all living cells. In contrast to humans, where an NAD-synthetase-independent recycling pathway exists, the biosynthesis of NAD^+ in *M. tuberculosis* is absolutely dependent on the activity of NAD-synthetase. Screening of compounds with proven inhibition of mycobacterial NAD-synthetase in a biochemical assay identified one promising compound inhibiting growth of *M. tuberculosis*. Thus, this new class of antibiotics raises hope for new possibilities to fight Tuberculosis.

Zusammenfassung

Mit mehr als 9 Millionen Neuinfektionen und annähernd 2 Millionen Todesfällen pro Jahr ist die Tuberkulose immer noch ein Problem mit globaler Tragweite. Der Erreger der Tuberkulose, *Mycobacterium tuberculosis,* gehört der Gruppe der langsam wachsenden, mit einem hohen GC-Anteil versehenen Gram-positiven Bakterien an. Mykobakterien im Allgemeinen sind mit einer einzigartigen äusseren Hülle ausgestattet, welche eine natürliche Schranke gegen die Aufnahme einer ganzen Reihe von chemischen Substanzen darstellt. Dies ist der Grund weshalb die Behandlung der Tuberkulose langwierig und komplex ist. Seit einiger Zeit wird ein Anstieg von Infektionen mit resistenten oder gar multiresistenten Tuberkulosestämmen beobachtet. Multiresistenz ist auf mehrere erworbene Mutationen in unterschiedlichen Genen zurückzuführen. Obwohl der Tuberkuloseerreger bereits vor einem Jahrhundert entdeckt und beschrieben wurde, sind wir von einem vollständigen Verständnis der Infektion und der Virulenzmechanismen von *M. tuberculosis* weit entfernt. Nach einer Tröpfcheninfektion mit *M. tuberculosis* gelingt es dem Bakterium in den Makrophagen der Lungenalveolen zu überleben und teilweise für Jahrzehnte im Körper zu persistieren. Es wurde gezeigt, dass eine spezielle Klasse von Proteinen, die Lipoproteine; in verschiedenen Prozessen der Wirt-Pathogen Interaktionen beteiligt sind. Das Ziel dieser Studie ist 1) die Untersuchung des Transports von Lipoproteinen zur mykobakteriellen Zellwand sowie die Charakterisierung spezifischer Lipoproteine auf molekularer Ebene und 2) die Evaluation neuer antibiotischer Zielstrukturen und neuer Antibiotika sowie die Verstärkung der Wirkung bestehender Antibiotika.

Zur Untersuchung des Transports von Lipoproteinen zur Zellwand von Mykobakterien, wurden verschiedene Lipoproteine von *M. tuberculosis* in *M. smegmatis,* einem mykobakteriellen Modell-Organismus, exprimiert. Mit der Hilfe von subzellulären Fraktionierungstechniken konnten diese Reporterproteine entweder in der Cytoplasmamembran oder in der Zellwand lokalisiert werden. Lipoproteine enthalten in ihrer maturen Form ein universell konserviertes und lipidiertes Cystein. In dem sehr gut untersuchten, Gram-negativen Bakterium *Escherichia coli* werden Lipoproteine durch das Lol-System zur äusseren Membran transportiert und verankert. Ein Rückhaltesignal, ein Asp an der Position +2 nach dem Cystein bewirkt, dass bestimmte Lipoproteine in der Plasmamembran verankert bleiben. Da Mykobakterien kein dem Lol-system entsprechendes Transportsystem besitzen, ist es wahrscheinlich, dass ihre Lipoproteine auch ein anderes Transportsignal besitzen. In dieser Studie wurden durch gezielte Mutationen verschiedene artifizielle Reporterlipoproteine generiert, in *M. smegmatis* exprimiert und ihre Lokalisation mittels Fraktionierung untersucht.

Die Resultate dieser Experimente zeigen, dass eine Region von 10 Aminosäuren unmittelbar C-terminal des Cysteins der Lipobox als ein positives Transportsignal dient.

Die aktuelle Standardtherapie zur Behandlung der Tuberkulose besteht aus einer Kombination mehrerer Antibiotika der ersten Wahl. Mit dem gehäuften Auftreten mehrfach medikamentenresistenter Stämme von *M. tuberculosis*, rücken die Medikamente der zweiten Wahl immer mehr in den Fokus. Aufgrund ihrer zum Teil schwierigeren Verabreichungsmethode (Injektion) und ihren grösseren Nebenwirkungen gelten diese Medikamente als zweite Wahl. Eines dieser Antibiotika ist Ethionamid. Ethionamid wird als ein Vorläufer verabreicht und muss, um seinen antibakteriellen Effekt zu entfalten, im Bakterium aktiviert werden. Dieser Vorgang wird von dem mykobakteriellen Enzym EthA katalysiert, dessen Expression von seinem natürlichen Repressor EthR vermindert wird. Durch systematisches Screening einer Sammlung chemischer Substanzen wurde 2-Phenylethyl-butyrat (2-PEB) entdeckt, welches die Fähigkeit hat, EthR von dem *ethA*/*ethR*-Operon abzulösen. Die hier vorliegende Studie zeigt mittels Medikamenten-Suszeptibilitätstests, dass die Kombination von 2-PEB und ETH einen synergistischen inhibitorischen Effekt auf das Wachstum von Mykobakterien hat, sogar auf Stämme mit Antibiotikaresistenzen. Durch die Zugabe von 2-PEB konnte somit die minimale Hemmkonzentration gesenkt werden.

Der letzte Teil dieser Arbeit widmet sich neuen möglichen Antibiotika zur Behandlung der Tuberkulose. Es handelt sich hierbei um eine Klasse von NAD-Synthetase Inhibitoren. NAD^+ ist ein Coenzym, welches an Redox-Reaktionen in allen lebenden Zellen beteiligt ist. Im Gegensatz zu den Menschen, welche einen Recyclingweg für NAD^+ besitzen, ist die Synthese von NAD^+ in Bakterien vollständig von einer funktionellen NAD-Synthetase abhängig. Substanzen, welche in einem biochemischen Test die *M. tuberculosis*-NAD-Synthetase inhibierten, wurden bezüglich ihrer Fähigkeit untersucht, das Wachstum von *M. tuberculosis* zu hemmen. Eine der getesteten Substanzen zeigt eine vielversprechende Wirkung gegen *M. tuberculosis*. Diese neue Klasse von Antibiotika gibt Anlass zur Hoffnung im Kampf gegen die Tuberkulose.

Introduction

Tuberculosis

Tuberculosis is a major cause of death around the world, with 9.2 million new cases and 1.7 million deaths occuring in 2006, which is the highest rate claimed by a single bacterium (WHO, 2009). The causative agent of this disease, spreading among infected humans as much as one-third of the world's population, is *Mycobacterium tuberculosis*, an acid-fast bacillus that is transmitted primarly via the respiratory route. Although most organs can be infected with *M. tuberculosis*, the disease usually is manifested in the lungs. In most cases infections remain clinically asymptomatic. It is estimated that only 5-10% of persons infected with *M. tuberculosis* present with active disease (Porcelli, 2008). Infected persons who do not develop the disease are not contagious, but the infection can reactivate years later, resulting in active Tuberculosis. The risk of reactivation is increased in immunocompromised persons, including persons co-infected with HIV (Algood *et al.*, 2003). The reasons for the pathogens' extraordinary success are diverse: it evades the immune system by parasitizing the macrophages of its host, it has a thick waxy cell wall and therefore is resistant to different kinds of mechanical and chemical stress and it is slow-growing which makes antibiotic treatment complicated and lengthy.

The recommended standard treatment for Tuberculosis is a regimen of four out of five first line drugs (isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin) for initial two months, followed by a four months treatment with isoniazid and rifampicin. During the first phase with four different drugs, replicating bacteria are killed, sputum usually becomes sterile and patients become non-infectious. The following four months phase with two antibiotics eradicates the remaining non-replicating bacteria. Given the length, complexity and adverse event profile of this drug treatment, patient adherence can be difficult to maintain (Goodman & Lipman, 2008). Inadequate drug therapy selects for *M. tuberculosis* mutants, leading to acquired drug resistance. *M. tuberculosis* is naturally resistant to several antibiotics, due to the presence of hydrolytic and drug-modifying enzymes, drug efflux systems and the highly hydrophobic cell wall. Acquired drug resistances led to multidrug- and even extensively drug-resistant strains of *M. tuberculosis* during the 1990s (Ducati *et al.*, 2006; Jassal & Bishai, 2009). Multidrug-resistant strains of *M. tuberculosis* (MDR-TB) are defined by a resistance to the two most efficient drugs isoniazid and rifampicin (Böttger & Springer 2008). The term extensively drug-resistant TB (XDR-TB) is defined as MDR-TB further showing resistance to

a fluoroquinolone and at least one second-line injectable agent (amikacin, kanamycin and/or capreomycin) (WHO, 2007).

An additional reason for the pathogens spread is the HIV/AIDS epidemic over the past few decades. Any condition which perturbs protective host immunity to *M. tuberculosis* leads to an increased risk to develop active disease. The extent to which this occurs depends on the degree of disruption of the hosts' immune system and the number of people affected by such a condition. Rates of TB infection are therefore high in those countries in which HIV is prevalent, mainly in sub-Saharan Africa and Asia (Goodman & Lipman, 2008).

The most widely used vaccine throughout the world with an estimated number of more than 3 billion people being vaccinated is BCG (Bacille Calmette-Guérin) against Tuberculosis. After 231 passages of *Mycobacterium bovis*, the causative agent of bovine Tuberculosis, Albert Calmette and Camille Guérin observed a reduction in the virulence in animals through this period. When infants were given this vaccine, it provided a reduction in mortality by 90% (Bloom, 1994). BCG nowadays still is the only vaccine available against Tuberculosis but there are serious doubts about its efficacy. It has been shown to be effective against disseminated and meningeal Tuberculosis in young children. However, the protective effect against adult pulmonary TB varies dramatically from 80 to 0% (Gupta *et al.*, 2007). There have been recent advances in development of new vaccines against Tuberculosis: DNA vaccines, recombinant BCG vaccines, Sub-unit (protein and peptide) vaccines and living, attenuated vaccines. Some of the new approaches already have entered clinical evaluation and some of them showed promising results. But nevertheless, a new licensed vaccine might be available not before the next decade (Gupta *et al.*, 2007; Lambert *et al.*, 2009).

Pathogenesis

Infection with *Mycobacterium tuberculosis*, the causative agent of Tuberculosis, follows a relatively well-defined sequence of events. After being inhaled as droplets from the atmosphere, the bacteria are phagocytosed by alveolar macrophages and induce a localized proinflammatory response. This immunological reaction entails the recruitment of mononuclear cells which are building blocks for granuloma. This lesion, also called tubercle, which defines the disease, consists of infected macrophages in the center. They are surrounded by foamy giant cells, macrophages and lymphocytes, corresponding to the periphery of this structure. The activated macrophages restrict growth of the bacteria and therefore end the primary infection, commonly without any symptom presentation (Ducati *et*

al., 2006; Russell, 2001). Typical TB symptoms are weakness, fever, weight loss, night sweat, chest pain, respiratory insufficiency and cough.

In most cases, the bacteria coexist within the host in a dormant form of infection, though still representing a large bacterial reservoir among infected individuals. Only 10% of the people infected with *M. tuberculosis* develop an active TB during their life-time. Immunocompromised individuals, e.g. with an HIV-coinfection, malnutrition or old-age, are highly susceptible for a reactivated TB. As a consequence, the granuloma are not under the control of the cellular immune system anymore. Therefore, the number of lesions and the number of bacteria increases, leading to necrosis in the lung-tissue and cavity formation. Due to the lesions, the bacteria are able to spread by productive cough containing aerosols with a massive load of infectious bacteria (Hunter *et al.*, 2007). It has been shown in animal models that 1 to 10 tubercle bacilli are sufficient to cause an infection (Bloom & Murray, 1992). Besides being able to destroy the lung parenchyma, about 15% of the patients with an active disease develop miliary or extra-pulmonary TB. Because of the excessive growth, the bacteria start invading the blood stream and disseminate to various parts of the body. The disease then spreads among others to the pleura, lymph nodes, liver, spleen, bones and joints. The extraordinary success of *M. tuberculosis* is characterized by the ability of parasitizing the macrophages of its host. In contrast to other bacteria, pathogenic mycobacteria are able to manipulate the phagosome in a way that they are residing in and prevent the normal maturation of this organelle into an acidic, hydrolytic compartment (Russell, 2001, Vergne *et al.*, 2004).

It has long been recognized on clinical and epidemiologic facts that *M. tuberculosis* produces two types of disease, primary and postprimary Tuberculosis. This has recently been confirmed with genetic studies demonstrating that susceptibility to primary and postprimary Tuberculosis is governed by different genes (Alcais *et al.*, 2005). The initial infection of a person with *M. tuberculosis* produces primary Tuberculosis. The infection begins when the bacteria are inhaled into the lungs where they induce a granulomatous inflammatory response. The organisms rapidly spread from the lung to lymph nodes and hematogenously throughout the body. The infection is typically arrested and the lesions heal within 6-8 weeks with the development of effective cell-mediated immunity (Hunter *et al.*, 2007).

Postprimary, also known as secondary or adult Tuberculosis is any disease that develops after a state of immunity has been established. It may arise either from reinfeciton with new

organisms or reactivation of dormant ones. Approximately 80% of all clinical cases and nearly all transmissions of infection are due to postprimary Tuberculosis. Postprimary Tuberculosis differs from primary Tuberculosis in a way that it requires a strong immune response, is confined to the lung, does not spread to lymph nodes or distant sites and does not heal. It is characterized by cavities in the lung that produce massive numbers of organisms that are coughed into the environment (Hunter *et al.*, 2007).

Mycobacteria

M. tuberculosis belongs to the genus *Mycobacterium*, which comprises more than 130 species, among others *M. bovis* BCG, the vaccine strain which is used against tuberculosis, *M. leprae*, the causative agent of leprosy, and *M. smegmatis*, a non-pathogenic mycobacterial model organism. Mycobacteria are aerobic, straight or slightly curved rod-shaped Gram-positive bacteria with about a size of 3 x 0.3 µm. They are nonmotile, nonsporing, without conidia. Mycobacteria stain usually weakly Gram-positive and are acid-alcohol fast. Colonies are often yellow or orange with a dull and rough surface. A rough differentiation of Mycobacteria can be made based on the growth rate: *M. tuberculosis* is a slow growing organism with a generation time of 20 to 24 hours; *M. smegmatis* belongs to the group of rapid growing mycobacteria with a generation time of two to three hours. The complete genome sequencing of *M. tuberculosis* H37Rv, the best characterized laboratory strain of *M. tuberculosis*, showed that it consists of approximately 4000 genes and has a high G+C content of 65.6% (Cole *et al.*, 1998). The genome is rich in repetitive DNA, including insertion sequences in intergenic or non-coding regions, frequently close to tRNA genes. An additional unique feature is the high frequency of genes involved in fatty acid metabolism. About 250 enzymes are either involved in lipolysis, required for bacterial survival inside its host, or lipogenesis, mainly for cellular envelope synthesis (Ducati *et al.*, 2006).

The cellular envelope of *Mycobacterium spp.* is rather complex and consists of three major structures: the cytoplasmic membrane, the outer layer and the capsule-like structure. Although this structure resembles the cell envelope of Gram-negative bacteria (with the three layers: cytoplasmic membrane, periplasm and the outer membrane), *Mycobacteria spp.* phylogenetically belong to the group of (GC-rich) Gram-positive bacteria. The cytoplasmic membrane of mycobacteria is composed by phospholipids, as there are phosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. A major cytoplasmic membrane component, which is restricted to bacteria of the Actinomycetes group are phosphatidylinositol mannosides (PIMs). Additionally, PIMs form the lipid base of lipoarabinomannan (LAM), one of the components of the mycobacterial cell wall (Figure 1).

The adjacent cell wall skeleton mainly consists of a mycolyl-arabinogalactan-peptidoglycan complex. In contrast to most other bacteria, the peptidoglycan-layer of mycobacteria contains *N*-glycolylmuramic acid instead of *N*-acetylmuramic acid (Brennan & Nikaido, 1995). As an additional unique feature, the cell wall core of mycobacteria is composed of arabinogalctan which is attached to the peptidoglycan through a phosphodiester link. Like corynebacteria, nocardia and rhodococci, the cell envelope of mycobacteria is interspersed with mycolic acids. They are high-molecular-weight fatty acids which are bound to the arabinogalactan cluster by ester bonds. Compared to the other above mentioned bacteria, mycobacteria have the largest mycolic acids (C70 to C90) and contain double bonds or cyclopropane rings (Brennan & Nikaido, 1995). By hydrophobic interactions, the mycolic acids interact with free glycolipids, phenolic glycolipids and glycopeptidolipids.

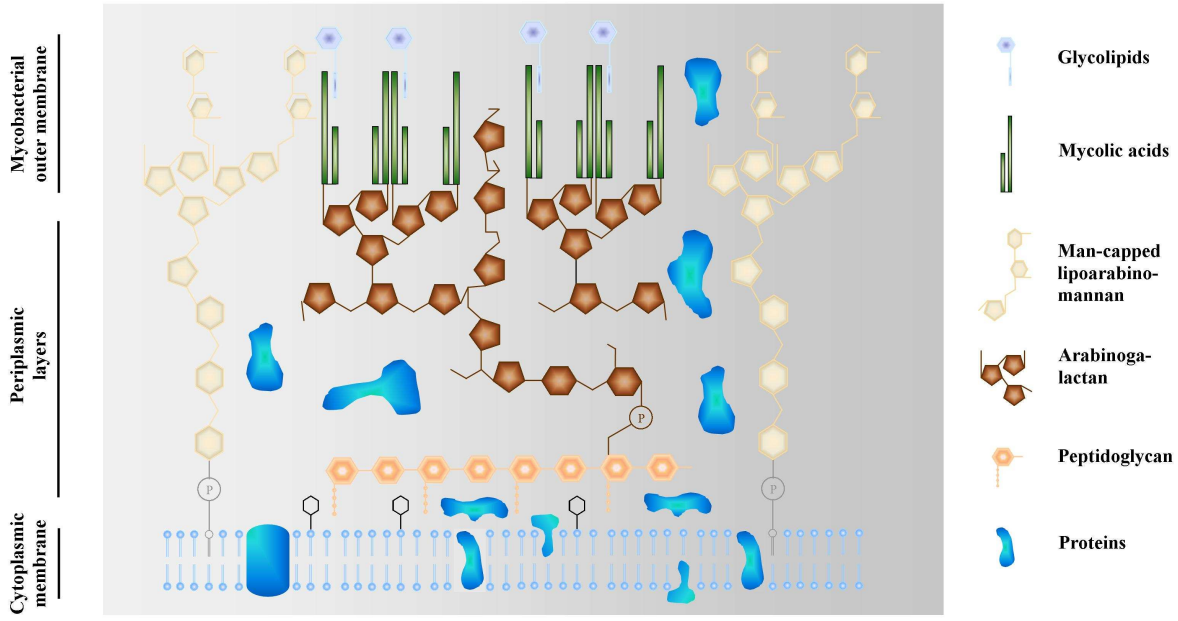

Figure 1: Schematic representation of the cell wall of *M. tuberculosis* (adapted from Besra et al. (Besra, 1994))

LAM are glycosylated phosphatidylinositol analogues, composed of a phosphatidyl-inositol anchor, a D-mannan polymer, D-arabinose chains and capping motifs at the nonreducing termini of the arbinose residues. Interestingly, the nonpathogenic *M. smegmatis* lacks mannose caps which have been detected in *M. tuberculosis*. LAMs are supposed to span the cell wall skeleton, therefore they can be regarded as a part of the cell wall even though they are attached to the cytoplasmic membrane by a phosphatidylinositol anchor (Brennan & Nikaido, 1995; Daffe & Draper, 1998). *M. tuberculosis* LAM has been shown to inhibit

phagosomal maturation by inhibition of Ca^{2+} rise. Nonpathogenic mycobacteria lacking the mannose caps of LAM are not able to inhibit the Ca^{2+} rise (Vergne *et al.*, 2004). The outermost structure of the mycobacterial cell envelope, the capsule-like structure consists of polysaccarides, proteins, free lipids such as waxes and glycolipids. Even though mycobacteria belong to the group of Gram-positive bacteria, recent investigations have shown that they possess an outer-membrane-like structure (Hoffmann *et al.*, 2008), which resembles Gram-negative bacteria with an outer membrane. Overall, the complex and dense architecture of the mycobacterial cell envelope turns it into an efficient permeability barrier, which is a necessary but not sufficient factor for resistance to a broad variety of antimicrobial agents.

Drug Resistance of Mycobacterium tuberculosis

Mycobacterium tuberculosis is naturally resistant to several antibiotics. In contrast to many other pathogenic bacteria, plasmid-mediated resistance has never been observed in *M. tuberculosis*. Resistant phenotypes are caused by chromosomal mutations, e.g. nucleotidic insertions, deletions or substitutions, in different genes (Ducati *et al.*, 2006). Shortly after the first drugs against Tuberculosis have have been introduced, resistance to these drugs was observed in clinical isolates of *M. tuberculosis* (Johnson *et al.*, 2006). All first-line drugs being used nowadays have been launched about a half a century ago (streptomycin (STR, launch date 1948), isoniazid (INH, 1952), pyrazinamide (PZA, 1954), ethambutol (EMB, 1962) and rifampicin (RIF, 1963)) (Harper, 2007). With the rise of MDR- and XDR-TB, special attention is paid to second-line drugs like fluorquinolones (e.g. ofloxacin (OFL) or ciprofloxacin (CIP)), ethionamide (ETH) or aminoglycosides like amikacin (AMK) or kanamycin (KAN). These drugs are usually more toxic and less effective than first-line drugs and therefore prolong the overall treatment up to 9 or even more months and besides causing severe side effects, are difficult in handling concerning patients' compliance. Table 1 gives an overview over the most frequent mutations causing drug-resistances for first- and second-line drugs against TB.

	Drug	Gene locus	Gene product	Most frequently mutated codons associated with resistance	Drug target/action
First line drugs	INH	*katG*	Catalase peroxidase	Ser315Thr	Activator of prodrug
		inhA	Enoyl-ACP recuctase	Promoter mutations:-24G/T, -16A/G, -15C/T, -8T-G/A. Structural gene mutations: Ile16Thr, Ile21Thr/Val, Ile47Thr, Val78Ala, Ile95Pro, Ser94Ala	Cell wall biosynthesis; final target is InhA which is involved in mycolic acid synthesis.
		ahpC	Alkyl hydroperoxide reductase	Promoter mutations: -46G/A, -39C/T	
		kasA	β-ketoacyl-ACP synthase	Asp66Asn, Gly269Ser, Gly312Ser, Phe413Leu	
		ndh	NADH dehydrogenase	Thr110Ala, Arg268His	
	RIF	*rpoB*	RNA polymerase	Ser531Leu, His526Tyr, Asp516Val	Transcription; RIF inhibits the β-subunit of the bacterial RNA polymerase.
	PZA	*pncA*	Pyrazinamidase	No hotspots	Activator of prodrug. Final target is the cell membrane; PZA lowers the pH intracellularly and thereby inhibits fatty acid synthesis
	EMB	*embB*	Arabinosyl transferase	Met306Leu/Val/Ile, Gly406Asp/Ser/Arg	Cell wall biosynthesis; inhibits arabinosyl transferase
	STR	*rrs*	16S RNA	491C/T, 513A/C-T, 906A/C	Translation, protein synthesis; interaction of STR with 16S rRNA
		rpsL	Ribosomal protein S12	Lys43Arg/Thr, Lys88Gln/Arg	
Second line drugs	Fluoroquinolones (CIP, OFL)	*gyrA*	DNA-gyrase	Ala90Val, Ser91Pro, Asp94Asn/His/Gly/Tyr/Ala	DNA metabolism; fluoroquinolones inhibit the DNA gyrase and DNA topoisomerase
		gyrB		Asn538Asp	
	Aminoglycosides (KAN, AMK)	*rrs*	16S rRNA	1401A/G, 1402C/T-A, 1408A/G	Translation, protein synthesis; binding of aminoglycosides to ribosomes disturb elongation of the peptide chain and induce misreading
	ETH	*inhA*	Enoyl-ACP recuctase	Promoter mutations:-24G/T, -16A/G, -15C/T, -8T-G/A. Structural gene mutations: Ile16Thr, Ile21Thr/Val, Ile47Thr, Val78Ala, Ile95Pro, Ser94Ala	*ethA* is the activator of the prodrug. Final target is InhA which is involved in mycolic acid synthesis; therefore, cell wall biosynthesis is inhibited by ETH
		ethA	Flavin monooxygenase	Gly43Cys, Asp58Ala, Ile338Ser, Gly385Ser	

Table 1: Properties of resistance to various anti-TB drugs

First-line drugs

Isoniazid. INH-resistance mainly has been linked to the following genes: *katG*, *inhA*, *ahpC*, *kasA* and *ndh*. INH is a pro-drug and has to be activated within mycobacteria. Mutations in *katG*, which encodes the associated activator catalase peroxidase KatG, are a main reason for resistance. Most *katG* mutations have been found between codon 138 and 328, the most common cause of resistance is the Ser315Thr mutation. This mutation is observed in 30-60% of INH resistant isolates (Slayden & Barry, 2000). One of the targets of INH is InhA, an enoyl-acyl carrier protein (ACP) reductase, which is a part of the fatty acid synthase type II system (FASII). The FASII is responsible for the synthesis of mycolic acids, the essential components of the unique mycobacterial cell wall. Two different types of mutations confer INH-resistance: Mutations in the promoter-region of the *mabA*-*inhA*-operon (-24G/T, -16A/G, -15C/T and -8TG/A) and mutations in the *inhA*-gene (Ile16Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro). The mutations in the promoter-region lead to an overexpression of InhA and thus cause a low level resistance to INH. Mutations in the target gene itself result in reduced drug binding and therefore less efficient mycolic acid synthesis. Although these mutations in the structural *inhA*-gene are associated with INH resistance, they are not frequently reported in clinical isolates. *kasA* is, like *inhA*, involved in the mycolic acid synthesis. The gene encodes for a β-ketoyl-ACP synthase. Four different mutations leading to amino acid substitutions have been reported (Table 1). These mutations have been described to confer low-level INH-resistance (Johnson *et al.*, 2006). The above mentioned mutation in *katG* (Ser315Thr) often is associated with an increase of alkyl hydroperoxide reductase AhpC which is able to detoxify damaging organic peroxides. Five mutations in the related promoter (Table 1), leading to an overexpression of AhpC, have been described. As AhpC has a detoxifying effect, it contributes in an indirect manner to INH-resistance. It is supposed to compensate for decreased *katG*-dependent catalase activity and does not provide protection to the antibiotic itself. To exert its antibiotic activity, INH forms a covalent adduct with NAD. Mutations in *ndh*, the gene encoding for NADH-dehydrogenase, cause defects in the enzymatic activity and therefore result in a depletion of NAD. Hence, mutations in the *ndh*-gene contribute to INH-resistance in an indirect way. The most prominent mutations are listed in Table 1.

Rifampicin. Together with INH, RIF forms the backbone for the treatment of TB. RIF binds to the β-subunit of the mycobacterial RNA-polymerase. The interaction of RIF with this subunit, which is encoded by *rpoB*, inhibits transcription and thereby kills bacteria. Resistance to RIF is confered by manifold mutations and short in frame deletions in the *rpoB*-

gene with the most prominent changes in codons Ser531Leu, His526Tyr and Asp516Val. More than 70% of RIF-resistant isolates are characterized by these mutations (Ramaswamy & Musser, 1998).

Pyrazinamide. PZA is thought to kill mycobacteria by inactivation of a fatty acid synthase through lowering the intracellular pH (Rivers & Mancera, 2008). Like INH, PZA is a prodrug and has to be activated by pyrazinamidase, encoded by *pncA.* PZA resistance is caused by all kinds of mutations affecting *pncA*-dependent pyrazinamidase activity, a non-essential enzymatic function. Of note, the antituberculosis activity of PZA is highly specific for *M. tuberculosis* as e.g. *M. bovis* is naturally resistant due to a *pncA*-point mutation (169G/C).

Ethambutol. EMB inhibits the cell wall biosynthesis by interaction with an arabinosyl transferase, encoded by *embB.* About 70-90% of all EMB-resistant isolates have been shown to carry a variety of mutations in codon 306 (Table 1.), resulting in three different amino acid substitutions. Nevertheless, one third of EMB-resistant strains have a wildtype-form of *embB.* The fact that homologous arabinosyl transferases have been found (*embCAB*) (Telenti *et al.* 1997) might explain this uncertainty.

Streptomycin. By interaction with the 16S rRNA and S12 ribosomal protein, STR causes misreading of mRNA and inibition of protein synthesis. Mutations in the two genes *rrs* (16S rRNA) and *rpsL* (S12 ribosomal protein) are related to STR resistance. Several mutations in a loop region of the 16S rRNA, which is a part of the aminoacyl-tRNA binding site, has been shown to confer STR resistance (Table 1). Amino acid substitutions at two positions in the *rpsL* gene were detected to be responsible for high level resistance to STR (Springer *et al.*, 2001).

Second line drugs

Fluoroquinolones. CIP and OFL, which belong to the group of fluoroquinolones target and inactivate DNA gyrase and a type II DNA topoisomerase. DNA gyrase is encoded by *gyrA* and *gyrB,* both of which do have a quinolone resistance-determining region (QRDR), with a size of 320 bp in case of *gyrA* and 375 bp in *gyrB* (Table 1, Johnson *et al.* 2006).

Aminoglycosides. The two aminoglycosides KAN and AMK bind to bacterial ribosomes and interfere with decoding of mRNA. Like in STR-resistant strains, mutations in *rrs* are responsible for resistance to AMK and KAN, but are located at different sites (Table 1).

Ethionamide. ETH is a pro-drug, which needs to be activated by the mycobacterial Baeyer-Villiger monooxygenase EthA to exert its antimicrobial activity by inhibiting the enoyl-ACP reductase InhA. Like INH, ETH does not bind directly to InhA but forms a covalent adduct with NAD instead. This ETH-NAD adduct inhibits InhA efficiently (Wang *et al.*, 2007). InhA, which is also the target of INH, is part of the fatty acid synthase type II system (FASII) which synthesizes mycolic acids, the essential components of the unique mycobacterial cell wall. The expression of *ethA* is under control of its natural repressor EthR (Baulard *et al.*, 2000) which contributes to an increased intrinsic resistance and therefore ETH-based Tuberculosis therapy is often not that effective, even when prescribed at high hepatotoxic doses (Hollinrake, 1968). Acquired ETH reistance is due to i) mutations in the *ethA* coding region and ii) mutations in the promoter of the *mabA-inhA*-operon of the target-gene *inhA* (e.g. T-8C, T-8A, C-15T). Therefore, cross-resistance of the potent first-line drug INH and second-line drug ETH are common, as mutations in *ethA* are a frequent mechanism of ETH-resistance in INH-resistant *katG*-mutants (Table 1).

Future perspectives on drug resistance and drug development

The short overview of the most important antimycobacterial drugs clearly points out several issues. Enhanced efforts are needed for intensified investigations on drug resistance mechanisms, especially of second-line drugs, as they represent oftenly the last line of defense to fight emerging MDR- and XDR-strains of *M. tubeculosis*. The need for research on either new antibiotics with novel mechanisms of action or possibilities to enhance existent antibiotics becomes obvious. Additional challenges are the shortening of current treatment of Tuberculosis to improve patients' compliance and therefore embank the rise of drug-resistant strains of *M. tuberculosis,* and detailed research on resistance mechanisms of antimycobacterial drugs used nowadays.

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Lipoprotein synthesis in mycobacteria

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Microbiology (2007), **153**, 652-658

Abstract

Lipoproteins are a functionally diverse class of secreted bacterial proteins characterized by an N-terminal lipid moiety. The lipid moiety serves to anchor these proteins to the cell surface. Lipoproteins are synthesized as pre-prolipoproteins and mature by post-translational modifications. The post-translational modifications are directed by the lipobox motif located within the signal peptide. Enzymes involved in lipoprotein synthesis are essential in Gram-negative bacteria but not in Gram-positive bacteria. Inactivation of genes involved in lipoprotein synthesis attenuates a variety of Gram-positive pathogens, including *Mycobacterium tuberculosis*. The attenuated phenotype of these mutants indicates an important role of lipoproteins and lipoprotein synthesis in bacterial virulence. *M. tuberculosis*, the causative agent of tuberculosis, is one of the most devastating pathogens in the world. This article reviews recent findings on the synthesis, localization and function of lipoproteins in mycobacteria.

Introduction

Lipoproteins are a functionally heterogeneous class of proteins universally present in bacteria; typically between 1% and 3% of bacterial genomes encode lipoproteins (Babu *et al*., 2006; http://www.mrc-lmb.cam.ac.uk/genomes/dolop/). Lipoproteins represent a subgroup of secreted proteins characterized by the presence of a lipobox. The lipobox motif is located in the C-terminal part of the leader peptide and consists of four amino acids [LVI/ASTVI/GAS/C] (Babu *et al*., 2006). This motif functions as a recognition signal for lipid modification, which is made on the conserved and essential cysteine residue. Precursor lipoproteins are mainly translocated in a Sec-dependent manner across the plasma membrane and are modified subsequently. Recent investigations indicate that lipoproteins may also be translocated by the twin-arginine translocation (Tat) system (McDonough *et al*., 2005). However, this pathway presumably is of minor importance because the number of lipoproteins translocated via the Tat transporter is rather small. Modification of precursor proteins is mediated by the consecutive activity of three enzymes: phosphatidylglycerol–pre-prolipoprotein diacylglyceryl transferase (Lgt), prolipoprotein signal peptidase/signal peptidase II (LspA) and phospholipid–apolipoprotein N-acyltransferase (Lnt) (Fig. 1). While Lgt and LspA are universally present in bacteria, Lnt has been reported to be restricted to Gram-negatives (Wu, 1996). The lipid residue covalently linked to the conserved cysteine moiety is thought to allow for anchoring of proteins in biological membranes by means of hydrophobic interaction. In Gram-positive bacteria, cell-associated lipoproteins are found in the plasma membrane. In Gram-negative bacteria the majority of cell-associated lipoproteins are found in the outer membrane – only about 10% stay anchored in the plasma membrane. Lipoproteins may be divided into five general groups according to their function in adhesion and invasion, cell wall synthesis, nutrient uptake, degradative processes, and sensing and transmembrane signalling (Sutcliffe & Russell, 1995). Enzymes involved in lipoprotein synthesis (Lgt, LspA, Lnt) are essential in Gram-negative bacteria but not in Gram-positive bacteria (Wu, 1996; Leskela *et al*., 1999).

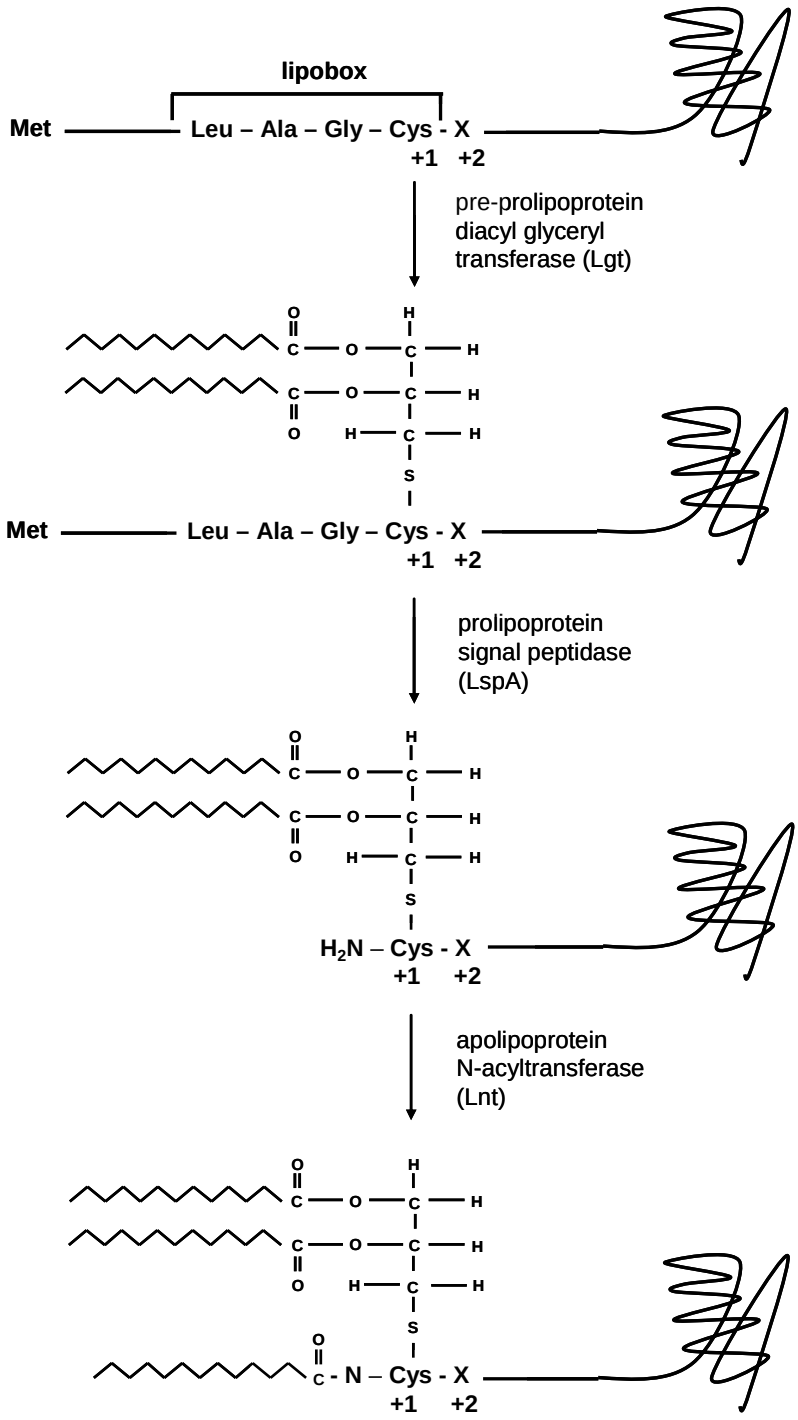

Figure 1: Biosynthesis of bacterial lipoproteins. Precursor lipoproteins are post-translationaly modified by phosphatidyl glycerol-prolipoprotein diacylglyceryl transferase (Lgt), lipoprotein signal peptidase (LspA). In Gram-negative bacteria and mycobacteria, lipoproteins are further processed by apolipoprotein *N*-acyltransferase (Lnt).

Mycobacteria belong to the group of GC-rich, Gram-positive bacteria, although the cell envelope of these bacteria is rather complex and in some respects resembles the cell envelope of Gram-negatives (Brennan & Nikaido, 1995). The lipid-rich outer layer in mycobacteria formed by mycolic acids and phospholipids is analogous to the outer membrane of Gram-negative bacteria. The genus *Mycobacterium* consists of more than 120 species (Rogall *et al*., 1990). The genome sequences of ten mycobacterial species have been determined or sequencing is near to completion (http://www.sanger.ac.uk; http://www.tigr.org). With respect to pathogenicity mycobacteria may be divided into obligate pathogens, opportunistic pathogens or nonpathogenic species. *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy, are major human pathogens. Opportunistic infections, in particular in patients with immune suppression, may be due to a variety of mycobacteria, e.g. *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium marinum*, *Mycobacterium kansasii* and *Mycobacterium chelonae*. *Mycobacterium smegmatis*, a non-pathogenic, fast-growing mycobacterium, has been established as a workhorse in mycobacterial genetics and biochemistry. Bioinformatic analyses suggest the presence of at least 48 lipoproteins but potentially twice as many in the genome sequence of *M. tuberculosis* (Sutcliffe & Harrington, 2004); similar numbers are found in other mycobacteria (Table 1). Experimental data concerning mycobacterial lipoprotein function are rare. Nearly half of the annotated *M. tuberculosis* lipoproteins do not share conserved domains with proteins outside the genus *Mycobacterium* and thus represent unique proteins of mycobacteria (Sutcliffe & Harrington, 2004). We here review recent findings on the synthesis, localization and function of lipoproteins in mycobacteria; emphasis is given to *M. tuberculosis* and *M. smegmatis*.

Table 1. Lipoproteins in representative mycobacterial genomes

Species	No. of predicted lipoproteins	No. of ORFs	Relative no.of lipoproteins (%)
M tuberculosis	48	3918	1.2
M. leprae	25	1604	1.6
M. avium	75	4350	1.7
M. marinum	97	5485	1.8
M. smegmatis	61	6776	0.9

LipoP was used as a tool to predict lipoprotein signal peptides (http://www.cbs.dtu.dk/services/LipoP/; Sierakowska et al., 2003). Protein sequences have been downloaded from the TIGR comprehensive microbial resource (www.tigr.org), except for *M. marinum* which has been downloaded from the Sanger Institute (www.sanger.ac.uk). The lower number of predicted lipoproteins in our search as compared to previous reports (e.g. Sutcliffe and Harrington, 2004) is due to the more restrictive LipoP algorithm.

Mycobacterial lipoprotein synthesis

Lgt. Lipoproteins are synthesized as pre-prolipoproteins and mature by post-translational modifications. Lipoprotein-modifying enzymes are membrane-integral proteins located in the plasma membrane. The first step of lipoprotein synthesis is conferred by Lgt, which adds a diacylglyerol residue to the thiol group of the universally conserved cysteine within the lipobox (Fig. 1). In *Escherichia coli*, the lipid moiety of lipoproteins is derived from the membrane lipid phosphatidylglycerol (Wu, 1996). Phosphatidylglycerol is not a homogeneous entity, as its acyl chains may be composed of 16–20 carbon atoms. In addition, the acyl chains may be saturated or unsaturated. So far, lipid moieties of mycobacterial lipoproteins have not been determined at the molecular level. However, in analogy to *E. coli* it may be hypothesized that the lipid moieties reflect the composition of mycobacterial membrane phospholipids. Therefore, despite identical protein moieties lipoproteins may vary to some extent.

Lipid modification of proteins can be demonstrated by labelling with radioactive palmitate and by detergent solubility. Early investigations exploited incorporation of [^{14}C] palmitic acid to demonstrate lipoprotein synthesis in *M. tuberculosis* (Young & Garbe, 1991). Radioactive labelling was also used to demonstrate protein lipidation in *M. smegmatis* (Kriakov *et al*., 2003). *M. tuberculosis* Lgt (Rv1614) is composed of 468 aa (mol. mass 50.4 kDa) and thus considerably larger than its *E. coli* homologue (291 aa). Compared to *E. coli* the *M. tuberculosis* Lgt, as well as Lgt of other members of the order Actinomycetales, is characterized by an additional C-terminal domain of unknown function. The isoelectric points of *M. tuberculosis* Lgt (pI 4.45) and *E. coli* (pI 9.66) differ greatly. The high pI of the *E. coli* enzyme is assumed to be important for interaction with acidic phospholipids by ionic as well

as hydrophobic interactions (Wu, 1996). The low pI of the *M. tuberculosis* Lgt enzyme is due to the unusually high aspartate and glutamate content of the C-terminal extension. In the *M. smegmatis* genome MSMEG3232 is annotated as Lgt. MSMEG3232 is composed of 612 aa (mol. mass 64.6 kDa) and like its *M. tuberculosis* homologue has a low pI (3.92). The functional consequences of the dramatic pI differences between the *E. coli* and the mycobacterial Lgt remain to be elucidated. In *M. smegmatis* a second ORF, MSMEG5388, is annotated as a putative Lgt. This ORF encodes a protein of 261 aa (mol. mass 31.0 kDa) with a pI of 9.38. However, a multiple sequence alignment revealed that the protein encoded by MSMEG5388 differs from the Lgt consensus sequence at several conserved residues (data not shown).

LspA. Lgt-modified prolipoproteins are further processed by LspA. LspA cleaves off the signal peptide directly in front of the modified cysteine. LspA knock-out mutants therefore accumulate prolipoproteins (Fig. 1). Due to the presence of the leader peptide, prolipoproteins have a slightly (2–3 kDa) higher molecular mass than the mature lipoproteins. Accumulation of prolipoproteins was demonstrated in *M. tuberculosis lspA* (Rv1539) (Sander *et al*., 2004; Banaiee *et al*., 2006) and recently in *M. smegmatis lspA* (MSMEG3181) knock-out mutants (M. Rezwan, A. Tschumi, T. Grau, S. Kuhn, P. Keller, B. Springer, E. C. Böttger & P. Sander, unpublished data). Inactivation of *M. tuberculosis* LspA by allelic replacement revealed an essential role of lipoprotein synthesis in the pathogenesis of *M. tuberculosis*. An *M. tuberculosis lspA* knock-out mutant exhibited reduced multiplication in the mouse macrophage cell line J774, complete absence of lung pathology and a 3–4 log reduced number of c.f.u. in a mouse model of tuberculosis infection (Sander *et al*., 2004). However, the molecular mechanisms underlying *M. tuberculosis lspA* attenuation remain to be determined.

Lipoproteins are potent agonists of TOLL-like receptor (TLR) 2, and extracts of an *M. tuberculosis* LspA knock-out mutant failed to induce a TLR2 response in the TLR2-reporter cell line HEK293. However, TLR-dependent activation in macrophages by entire mycobacteria or whole-cell lysates was not affected by LspA inactivation (Banaiee *et al*., 2006), indicating the existence of TLR ligands other than mature lipoproteins. The

redundancy of TLR agonists makes it unlikely that attenuation of the *lspA* knock-out mutant is due to alterations in TLR signalling[1].

Globomycin, a cyclic peptide produced by several Streptomyces species, is a potent and specific inhibitor of lipoprotein signal peptidases in different bacterial species. Due to the essentiality of lipoprotein-synthesizing enzymes, globomycin exerts a bactericidal effect in *E. coli* (Inukai *et al*., 1978). Inhibition of lipoprotein maturation by globomycin, as indicated by accumulation of precursor lipoproteins, has also been shown for Gram-positive bacteria (Harrington *et al*., 2000). Due to the severe attenuation of the *M. tuberculosis lspA* knock-out mutant, LspA has been proposed as a putative mycobacterial drug target and exploitation of globomycin as a prototype inhibitor for further drug development has been suggested (Sander *et al*., 2004). Promising antibacterial activity of globomycin derivatives has recently been demonstrated in Gram-positive bacteria (Kiho *et al*., 2004), but their activity towards mycobacteria remains to be determined.

Lnt. In Gram-negative proteobacteria, but not in Gram-positive bacteria, LspA-processed lipoproteins are further modified by Lnt (Wu, 1996). Lnt adds a third acyl residue to the amino group of the modified cysteine. *In vitro* and *in vivo* studies have indicated that any of the three major phospholipids in the *E. coli* cell envelope (phosphatidylethanolamine, phosphatidylglycerol, cardiolipin) can serve as an acyl donor (Wu, 1996). In *E. coli*, Lnt modification is a prerequisite for transport of lipoproteins across the periplasm. Based on the assumption that *N*-acylation of lipoproteins is required for transport into the mycolic acid layer, the presence of Lnt homologues in mycobacteria may be postulated. Multiple sequence alignments (data not shown) confirmed early *in silico* findings (Gurcha *et al*., 2002; Baulard *et al*., 2003) that Lnt homologues are present in mycobacteria. *M. tuberculosis* Rv2051c encodes a two-domain protein, where the N-terminal part shows similarity to *E. coli* Lnt. The C-terminal part of the protein encodes a polyprenolmonophosphomannose (Ppm) synthase, which transfers mannose from GDP-mannose to endogenous polyprenol phosphates, an important metabolic intermediate in the synthesis of the mycobacterial cell wall constituents lipomannan and lipoarabinomannan (LAM). The Lnt-homology domain of Rv2051c enhances the Ppm synthase activity of the C-terminal domain, while apo-lipoprotein *N*-acyltransferase

[1] Note added in proof: This assumption was confirmed recently by infection of TLR2$^{-/-}$mice, in which *M. tuberculosis lspA::aph* was also attenuated (**Rampini *et al*., 2008**. LspA inactivation in *Mycobacterium tuberculosis* results in attenuation without affecting phagosome maturation arrest. *Microbiology*, **154**,2991-3001)

activity remains to be demonstrated (Baulard *et al*., 2003)[2]. Besides the N-terminal domain of Rv2051c, ORF Rv2262 has lower but still significant homology to *E. coli* Lnt. Again, an enzymic function remains to be determined. In *M. smegmatis*, orthologues of the two domains of *M. tuberculosis* Rv2051c are encoded by two distinct ORFs, Msppm1 and Msppm2, of which Msppm2 (MSMEG3860) corresponds to Lnt. Thus, the *M. tuberculosis* Lnt ORF encodes a significantly larger protein (874 aa) compared to *M. smegmatis* (654 aa) and *E. coli* (512 aa). Lnt homologues have been found not only in mycobacteria but also in other actinobacteria, including *Streptomyces* and *Corynebacterium*. A comprehensive analysis of fully sequenced genomes of bacteria representing different phyla suggests that Lnt homologues are present in microorganisms with a complex cell envelope (unpublished observations) but not in ordinary Gram-positive bacteria.

The presence of Lnt homologues and the localization of lipoproteins in the mycobacterial cell wall (see below) suggest that a transport system for lipoproteins into the mycolic acid layer should exist – homologous or analogous to the *E. coli* Lol system. The *E. coli* Lol system consists of (i) an ABC-transporter complex of three proteins (LolC, LolD and LolE), which releases lipoproteins from the plasma membrane; (ii) a periplasmic chaperone (LolA); and (iii) a receptor in the outer membrane (LolB), which is itself a lipoprotein. *E. coli* lipoproteins are translocated to the outer membrane unless they possess a Lol-avoidance signal, i.e. an aspartate at position +2 (Narita *et al*., 2004). More complex lipoprotein transport signals may be present in other bacterial species (Schulze & Zuckert, 2006). Protein sequence alignments so far have not identified Lol homologues in *M. tuberculosis*. However, recent structural analysis of *M. tuberculosis* lipoprotein LppX revealed structural homologies to *E. coli* LolA and LolB, although the primary substrate seems to be phthiocerol dimycocerosate rather than lipoproteins (Sulzenbacher *et al*., 2006).

Lipoprotein localization

The function of a protein depends on its correct localization and vice versa. In Gram-positive bacteria, cell-associated lipoproteins stay anchored in the plasma membrane. In Gram-negative bacteria, the vast majority of cell-associated lipoproteins are released from the plasma membrane and are anchored to the outer membrane (Tokuda & Matsuyama, 2004). Despite recent progress in subcellular fractionation of mycobacteria (Mawuenyega *et al*.,

[2] Note added in proof: Lnt-dependent (Rv2051c and MSMEG3860) *N*-acylation of mycobacterial lipoproteins has recently been demonstrated (for details see chapters 3 and 4, **Tschumi *et al*.**, **2009**. Identification of Apolipoprotein *N*-Acyltransferase (Lnt) in Mycobacteria. *J Biol Chem*, **284**, 27146-27156. and **Bruelle et al**. 2010. Cloning, expression and characterization of *Mycobacterium tuberculosis* lipoprotein LprF. *Biochem Biophys Res Commun*, **391**, 679-684.)

2005; Rezwan *et al*., 2006), localization of mycobacterial lipoproteins has rarely been addressed. *M. tuberculosis* LprG and *M. smegmatis* PhoA were shown to be anchored in the cell envelope (Kriakov *et al*., 2003); likewise *M. tuberculosis* Mpt83 was shown to be cell surface associated by electron microscopy and fluorescence cytometry (Vosloo *et al*., 1997; Harboe *et al*., 1998). Proteomic analyses of fractionated *M. tuberculosis* extracts identified and located 28 putative lipoproteins (Mawuenyega *et al*., 2005). Six lipoproteins were found in the cell wall fraction, ten in the plasma membrane fraction and seven in the cytosolic fraction; five lipoproteins were observed in two or in all three fractions. Localization of mature lipoproteins in the cytosol is questionable and these lipoproteins may represent recently synthesized (pre-pro-) proteins not yet secreted. Subcellular fractionation of *lgt, lspA* and *lnt* mutants expressing recombinant proteins will help to elucidate the mechanisms underlying mycobacterial lipoprotein sorting.

Lipoproteins

In an excellent review Sutcliffe & Harrington (2004) discussed the function of individual *M. tuberculosis* lipoproteins in great detail. We therefore limit our discussion to recent findings. Most mycobacterial lipoproteins have been predicted by a bioinformatic approach, i.e. identification of the lipobox consensus sequence within a typical signal peptide (Sutcliffe & Harrington, 2004). Experimentally investigated lipoproteins often are immunodominant antigens. However, pleiotropic and seemingly antagonistic, i.e. pro- and anti-inflammatory effects, make it difficult to discern the mechanisms by which these proteins contribute to immunopathogenesis (Karakousis *et al*., 2004). Investigations with *M. leprae* LpK variants differing in the length of the peptide and the presence or absence of the lipid moiety revealed that both acyl residues and peptide sequences are required for elicitation of an immune response. The inability of a non-acylated 27 kDa antigen of *M. tuberculosis* to induce IFN-γ secretion corroborates the importance of the lipid moiety for inducing an immune response (Hovav *et al*., 2004).

Targeted gene inactivation of individual lipoprotein genes, transposon site hybridization mutagenesis and vaccination studies corroborate early findings on the overall importance of lipoproteins in the immunopathogenesis of infection with *M. tuberculosis*. Several puative lipoproteins, e.g. LppP (Rv2330c), LprO (Rv2290), LprK (Rv0173), LpqT (Rv1016c), LpqY (Rv1235), LpqZ (Rv1244), LprG (Rv1411) and LppX (Rv2945c), are required for optimal growth *in vivo* (Rengarajan *et al*., 2005; Sassetti & Rubin, 2003; Bigi *et al*., 2004). Some of these *M. tuberculosis* lipoproteins have homologues in mycobacteria, others represent

signature proteins for the *Corynebacterium*, *Mycobacterium*, *Nocardia* (CMN) subgroup of the actinobacteria (Gao *et al*., 2006) (Table 2).

Table 2. Distribution of lipoproteins in mycobacteria and sequence identities/similarities

Name	*M. tuberculosis**	*M. leprae* †	*M. avium* †	*M. marinum* ‡	*M. smegmatis* §
19kDa	LpqH	48/65	75/86	48/ 51	39/51
38kDa	PstS1	33/48	36/48 ?	32/ 46	34/49 ¶
PstS2	PstS2	62/77 ¶	77/85	66/ 77	45/56 ¶
PstS3	PstS3	77/86	78/86	77/ 83	48/61
LpqB	LpqB	87/92	86/92	82/87	72/81
LprF	LprF	30/48 #	29/51 #	65/ 72	32/48 #
LprJ	LprJ	34/56	66/85	71 /82	41/62
RpfB	RpfB	82/89	85/92	80/86	73/81
24kDa	LppX	76/85	34/52 #	73/ 85	30/47 #
LpqW	LpqW	79/85	81/88	72/77	74/82

* *M. tuberculosis* H37Rv lipoproteins were used as a query
† BLAST on http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi
‡ BLAST on http://www.sanger.ac.uk/cgi-bin/blast/submitblast/m_marinum
§ BLAST on http://tigrblast.tigr.org/cmr-blast
? Same ORF as identified by PstS2
¶ Same ORF as identified by PstS3.
# Same ORF as identified by LprG.

Examples of mycobacterial lipoproteins

The 19 kDa antigen (LpqH, Rv3763) of *M. tuberculosis* (see Table 2 for summary of lipoproteins discussed) is a glycosylated lipoprotein. Early on, this protein was shown to induce a TLR-2-dependent bactericidal response in macrophages (Thoma-Uszynski *et al*., 2001). Recently, the 19 kDa antigen was described as an adhesin, binding to the mannose receptor of THP-1 monocytic cells and thereby stimulating phagocytosis (Diaz-Silvestre *et al*., 2005). The 19 kDa antigen was shown to induce interleukin-1 and -12, and tumour necrosis factor-α (TNF-α) through TLR2- signalling in macrophages. Prolonged exposure to the 19 kDa lipoprotein inhibits IFN-γ production and major histocompatibility (MHC) class II expression. These findings suggest that, at least in part, persistent TLR2 signalling enables *M. tuberculosis* to evade T cell responses and persist as a long-term infection. An

M. tuberculosis 19 kDa knockout mutant was reported to be slightly attenuated in IFN-γ activated monocyte derived macrophages (Stewart *et al*., 2005).

Phosphate transport receptors (Pst): three homologues of the periplasmic ABC phosphate-binding receptor PstS of *E. coli* have been described in *M. tuberculosis* [PstS1 (Rv0934), PstS2 (Rv0932c) and PstS3 (Rv0928)]. Expression of these proteins increases under phosphate-limiting conditions. *M. tuberculosis* mutants deficient in PstS1 and PstS2 showed decreased c.f.u. in lungs and spleens of mice, indicating a role in virulence (Peirs *et al*., 2005). Vaccination of C57BL/6 mice with PstS3 DNA protected against challenge with *M. tuberculosis* (Romano *et al*., 2006). Compared to PstS3 vaccination, vaccination with PstS2 and PstS1-DNA induced only modest reduction in c.f.u. counts.

LpqB (Rv3244c) is a particularly interesting lipoprotein, as it is one of the 233 conserved signature proteins of the actinobacteria (Gao *et al*., 2006). *lpqB* is located immediately downstream of the two-component signal transduction system MtrAB. In this system MtrB is the transmembrane sensor-kinase and MtrA is the cytoplasmic response regulator. Lipoproteins have been shown to function as accessory proteins of sensor-kinase systems and it may be assumed that LpqB modulates signal sensing by MtrB (Hoskisson & Hutchings, 2006).

Lipoproteins LprF (Rv1368) and LprJ (Rv1690) exhibited protein–protein interactions with the histidine kinase KdpD in a two-hybrid screen. Activation of the Kdp signal transduction pathway appears to be the primary response to environmental osmotic stress in both *M. tuberculosis* and *M. smegmatis*. The histidine kinase domain of Kdp has been suggested to form ternary complexes with LprF and LprJ and it was speculated that these proteins function as ligand-binding proteins. Co-induction of LprJ with a cluster of genes involved in cell wall integrity suggests that LprJ is involved in this process (Boshoff *et al*., 2004). Alternatively, LprF or LprJ could function as accessory proteins as discussed for LpqB.

RpfB (Rv1009) is the only lipoprotein among the five resuscitation-promoting factor (Rpf) proteins of *M. tuberculosis*. Rpf proteins stimulate dormant cells to divide (Keep *et al*., 2006). They have structural homology to glycoside hydrolases and cleave peptidoglycan; however, the exact mechanism by which these proteins promote resuscitation remains elusive. Investigations in a mouse model of *M. tuberculosis* persistence and reactivation indicated that inactivation of *rpfB*, but not inactivation of *rpfA*, C, D or E, delayed reactivation, suggesting a unique role of RpfB in resuscitation (Tufariello *et al*., 2006). Whether this unique role of RpfB is related to the lipid anchor remains to be determined.

Investigation of LpqW and LppX revealed that these lipoproteins are key players in synthesis and transport of the unique components of the mycobacterial cell envelope. LppX (Rv2945c) is a lipoprotein involved in translocation of complex lipids, the phthiocerol dimycocerosates (DIM), to the outer membrane. Structural elucidation of LppX revealed the presence of a hydrophobic cavity suitable for binding the large lipophilic side chain of DIM (Sulzenbacher *et al*., 2006). Orthologues of LppX are only present in mycobacteria which synthesize DIM (e.g. *M. tuberculosis*), and are absent from DIM-negative mycobacteria (e.g. *M. smegmatis* or *M. avium*). An *M. tuberculosis* mutant deficient in LppX is attenuated in a mouse model of infection. Attenuation is associated with a failure to release DIM into the culture supernatant rather than reduced DIM synthesis (Sulzenbacher *et al*., 2006). LpqW (Rv1166) is a highly conserved, essential lipoprotein involved in the synthesis of cell wall components. Structural analyses suggest that LpqW is derived from substrate-binding proteins, which in mycobacteria and other micro-organisms of the subfamily *Corynebacterinae* (also producing LAM) has evolved to match the specific needs in the synthesis of the cell wall components phosphatidyl-myo-inositol mannoside (PIM) and LAM. LpqW acts at the branching point of the PIM/LAM pathway and converts the last common intermediate, the tetramannosylated phosphatidylinositol mannoside PIM4, into the LAM pathway (Kovacevic *et al*., 2006; Marland *et al*., 2006).

Conclusions

Lipidation of proteins is required for their anchoring and sorting to the cellular surface. Recent investigations point to the importance of lipoprotein-synthesizing enzymes as well as individual lipoproteins in the biology of mycobacteria. Mycobacterial lipoproteins are crucial for synthesizing the unique mycobacterial cell envelope, sensing of and protection from environmental stress and participation in host– pathogen interaction. Due to their contribution to virulence, lipoproteins and the enzymes of the lipoprotein synthesis pathway represent promising drug targets. In addition some lipoproteins confer a protective immune response and thus may qualify as subunit vaccines. Despite progress in lipoprotein research much remains to be learned with respect to the synthesis, localization and function of mycobacterial lipoproteins and their role in host–pathogen interaction.

Acknowledgements

This work was supported by a grant from the Swiss National Science Foundation (contract 3200-068488). We thank Erik C. Böttger for continuous support and critical comments on the manuscript.

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Addendum

Personal contribution to chapter 1

This Mini-Review gives a short overview of the lipoprotein biosynthesis pathway in mycobacteria, as well as of the knowledge about lipoproteins in *M. tuberculosis*. Lipoproteins are a class of secreted, membrane-anchored proteins characterized by a lipidated N-terminus. Precursor lipoproteins mature by three enzymatic modifications catalyzed by the integral membrane proteins Lgt, LspA and Lnt. Lgt attaches a diacylglycerol to the sulfhydryl group of the universally conserved cysteine in the lipoprotein recognition motif termed lipobox. Subsequently, LspA cleaves off the signal peptide and finally Lnt adds an acyl chain to the N-terminal cysteine. We observed by bioinformatic analysis the presence of 25 up to 97 lipoproteins in different mycobacteria. Out of the 48 putative lipoproteins in *M. tuberculosis*, only a minority of those is charaterized in detail.

My contribution as a second author to this manuscript was as follows:

Bioinformatic analysis

- Database search for putative lipoproteins in *M. tuberculosis*, *M. leprae*, *M. avium*, *M. marinum* and *M. smegmatis*
- Analysis of the distribution of selected lipoproteins in several mycobacteria and alignments to detect protein sequence identities and similarities
- Writing of the manuscript

Lipoprotein localization and transport in mycobacteria

Introduction

Bacterial lipoproteins comprise a subset of membrane proteins that are covalently modified with lipids at the N-terminal cysteine. The common feature of lipoproteins is the lipobox which is located in the C-terminal part of the singal peptide. The lipobox is rather highly conserved and consists of four amino acids [LVI][ASTVI][GAS]C (Babu *et al.*, 2006). Synthesized as precursors in the cytoplasm, lipoproteins are translocated across the cytoplasmic membrane mainly by the Sec translocation machinery. A recent publication reports that lipoproteins may also be translocated by the twin-arginine translocation system (Tat-system) (McDonough *et al.*, 2005). Lipoprotein maturation subsequently occurs on the periplasmic side of the cytoplasmic membrane by the consecutive action of the three enzymes Lgt (pre-prolipoprotein diacyl glyceryl transferase), LspA (prolipoprotein signal peptidase) and Lnt (apolipoprotein *N*-acyltransferase). As a first step, Lgt attaches a diacylglycerol residue to the thiol group of the universally conserved cysteine within the lipobox. Secondly, LspA cleaves off the signal peptide in front of the modified cysteine, followed by the attachment of a third acyl residue to the free amino group of the cysteine by Lnt (Rezwan *et al.*, 2007a). The lipoprotein biosynthesis pathway is very well characterized in the Gram-positive bacterium *Bacillus subtilis* (Tjalsma *et al.*, 1999) and the Gram-negative bacterium *Escherichia coli* (Tokuda & Matsuyama, 2004). While the first two enzymes involved in lipoprotein synthesis, Lgt and LspA, are universally present in bacteria, Lnt has been reported to be restricted to Gram-negatives (Tjalsma *et al.*, 1999). Although being considered as Gram-positive bacteria, the cell envelope of mycobacteria resembles the cell envelope of Gram-negatives.

In *E. coli*, a relatively small number of lipoproteins remain in the plasma membrane (Robichon *et al.*, 2005). The vast majority is located in the outer membrane. The release of an outer membrane associated lipoprotein from the cytoplasmic membrane and its transport is performed by the Lol-system, consisting of five components (LolABCDE) (Tokuda & Matsuyama, 2004). The ABC transporter LolCDE recognizes outer membrane-directed lipoproteins and dissociates them from the inner membrane. The LolCDE-lipoprotein complex passes the lipoprotein to LolA, a periplasmic carrier protein. Upon traversing the periplasm, the lipoprotein is transferred to the outer membrane receptor protein LolB - which is an outer membrane lipoprotein itself - and incorporates it into the outer membrane (Narita & Tokuda, 2006). The signal specificity for the transport in *E. coli* is the amino acid position

+2 and +3 after the highly conserved cysteine (position +1). Aspartate at position +2 functions as an inner membrane retention signal. Additional combinations of membrane retention signals have been found by systematic mutagenesis of the residue at position +2. According to these results, tryptophan, phenylalanine or proline in combination with asparagine at position +3 are sufficient to retain lipoproteins in the inner membrane (Seydel *et al.*, 1999; Terada *et al.*, 2001). In *Pseudomonas aeruginosa* it has been observed, that lysine and serine at positions +3 and +4 respectively cause inner membrane retention as well as several other combinations of amino acids at positions +2 to +4 (Lewenza *et al.*, 2008; Narita & Tokuda, 2007).

In order to investigate the mycobacterial lipoprotein biosynthesis pathway and possible transport conditions of mycobacterial lipoproteins, several heterogeneously expressed lipoproteins of *M. tuberculosis* have been expressed in different strains of *M. smegmatis* (*wild-type*, Δ*lgt*, Δ*lspA* and Δ*lnt*). Subcellular fractionation of these strains has been applied to examine the localization (either cytoplasmic membrane or cell wall) of lipoproteins. Additionally, multiple site directed mutagenesis of different lipoproteins has been applied to the amino acid sequences adjacent to the cysteine at position +1 in order to screen for possible transport signals.

Results

Database search for enzymes of the lipoprotein biosynthesis pathway. BLASTp search analysis has been performed to elucidate possible homologues of the enzymes involved in the lipoprotein biosynthesis pathway in the bacterial kingdom. Protein sequences of Lgt, LspA, Lnt and LolA, B, C, D and E of *E. coli* have been used as queries. Figure 1a shows the results of the BLASTp with Lgt (*E. coli*) as query. Lgt is widely distributed in Gram-negative bacteria (α-, β-, γ-, Δ-, ε-Proteobacteria, Spirochetes, Aquifex, Cytophaga, and Thermotoga), low GC Gram-positive bacteria (Firmicutes, e.g. Clostridia, Mollicutes and Bacilli) and high GC Gram-positive bacteria (Actinobacteria, e.g. Streptomyces, Nocardia, Corynebacteria and Mycobacteria). None of the 46 selected species shows an E-value below the given threshold of 10^{-4} (dashed line). *E. coli* strain K12 has been used as a positive control resulting in an E-value close to 0 (10^{-173}). LspA is as widely distributed as Lgt (Figure 1b). A protein similar to LspA can be found throughout all bacterial strains used in this setup, except in *Clostridium perfringens* str. 13. *E. coli* strain K12 as a positive control has an E-value of 10^{-83}. According to the results shown in Figure 1c, Lnt seems to be completely absent in low GC Gram-

positive bacteria but is detected in all strains of the group of high GC Gram-positive bacteria, including *Mycobacteria*. Figure 1d shows the results of the BLASTp with *E. coli*-LolA as a representative for the Lol-system. LolA can be found in several but not all Gram-negative bacteria. Besides *E. coli*, the Lol-system seems exist amongst others in *Haemophilus influenzae*, *Pseudomonas aeruginosa* or *Neisseria meningitidis*. A total of 8 out of 14 Gram-negatives do have homologues to LolA. Neither in low GC nor in high GC Gram-positives LolA has been detected. BLASTp has been performed for LolB, C, D and LolE as well (data not shown). LolB, C and E have not been found in Gram-positives, indicating, that there is no need for such a lipoprotein transport system (in case of low GC Gram-positives) because a lack of a periplasm, or different proteins have to be responsible for the transport across the pseudo-periplasm in case of Actinobacteria. Similar proteins to LolD are broadly found by BLASTp-search in all bacteria which can be ascribed to the highly conserved ATP-binding cassette-transporter domain (data not shown).

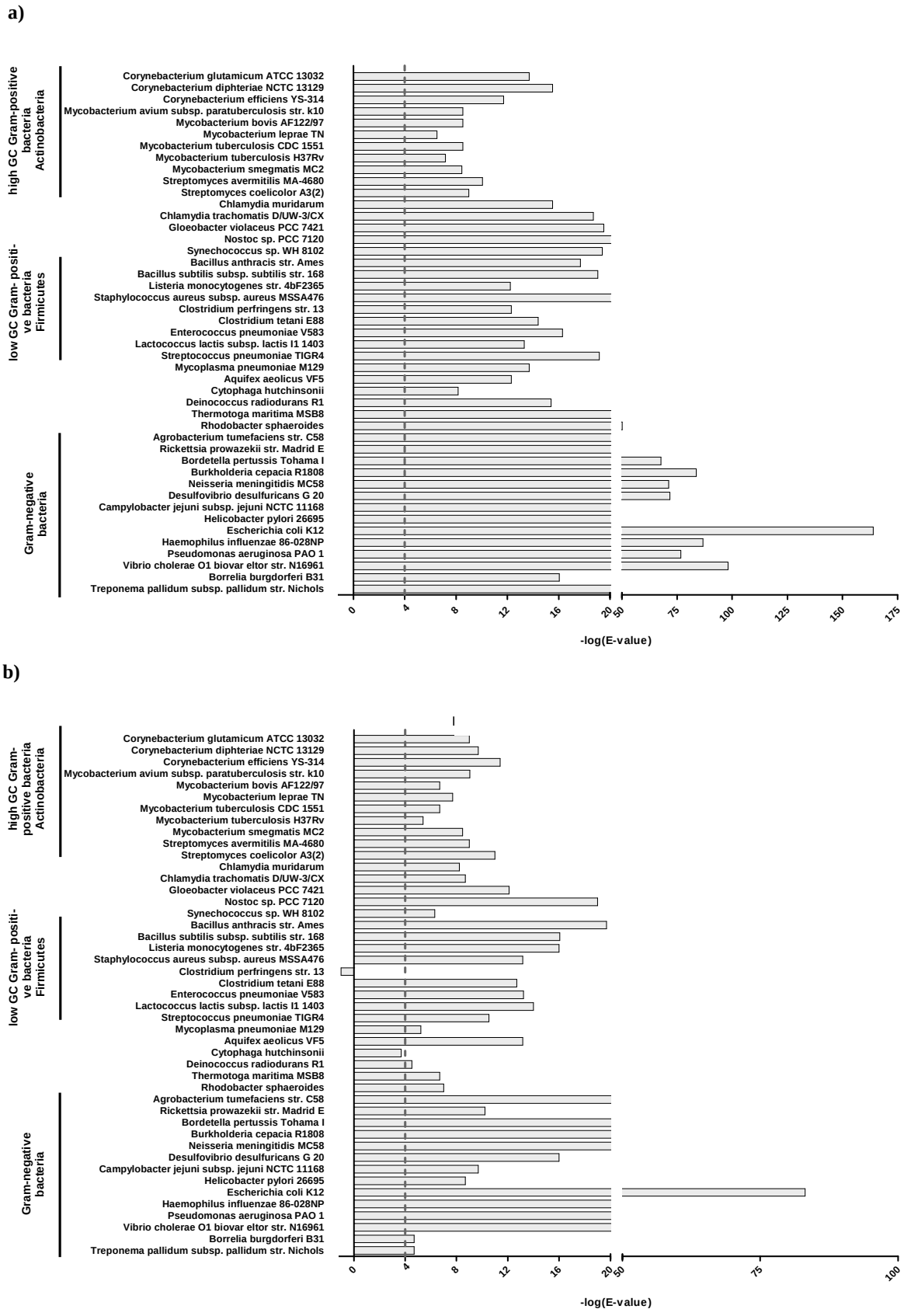
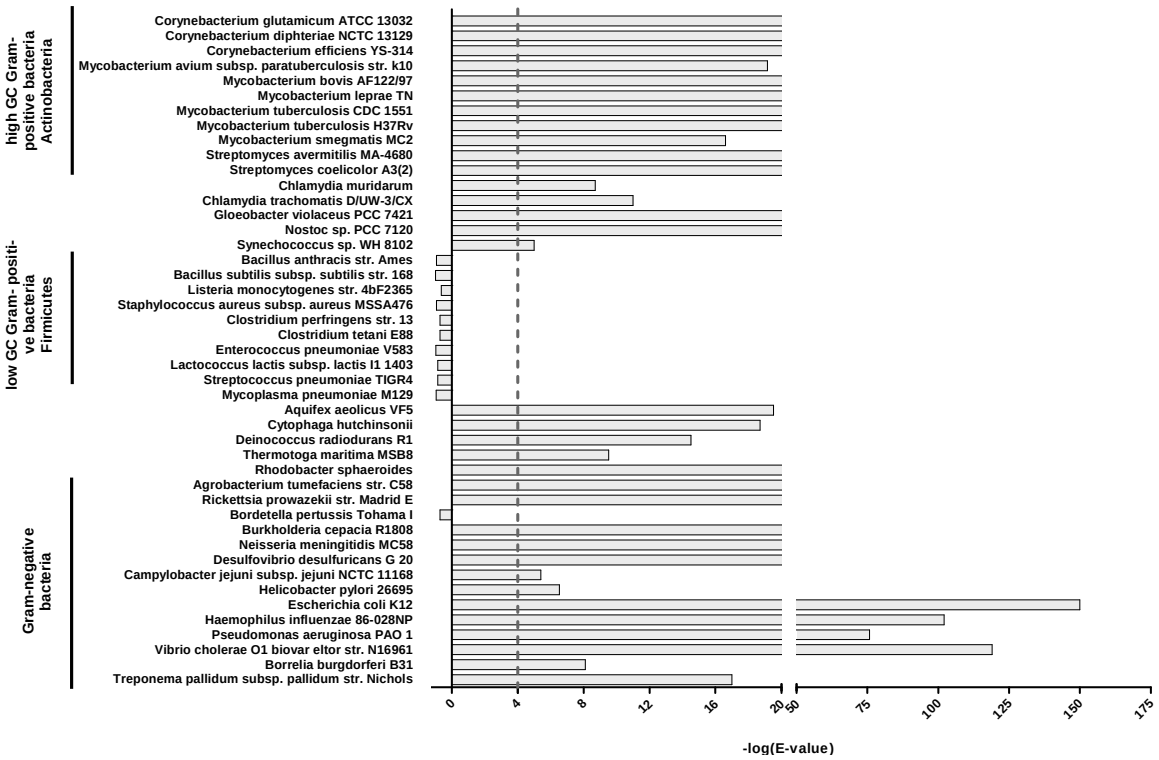


Figure 1. BLASTp search for lipoprotein modifying- and transport proteins. *E. coli* Lgt (a)) and LspA (b)) were used as queries to identify homologues on the National Center for Biotechnology Information BLASTp server. The sequence filtering option was switched off and the expect value was set at 10, the cut-off value set at 10^{-4} (dashed line).

c)



d)

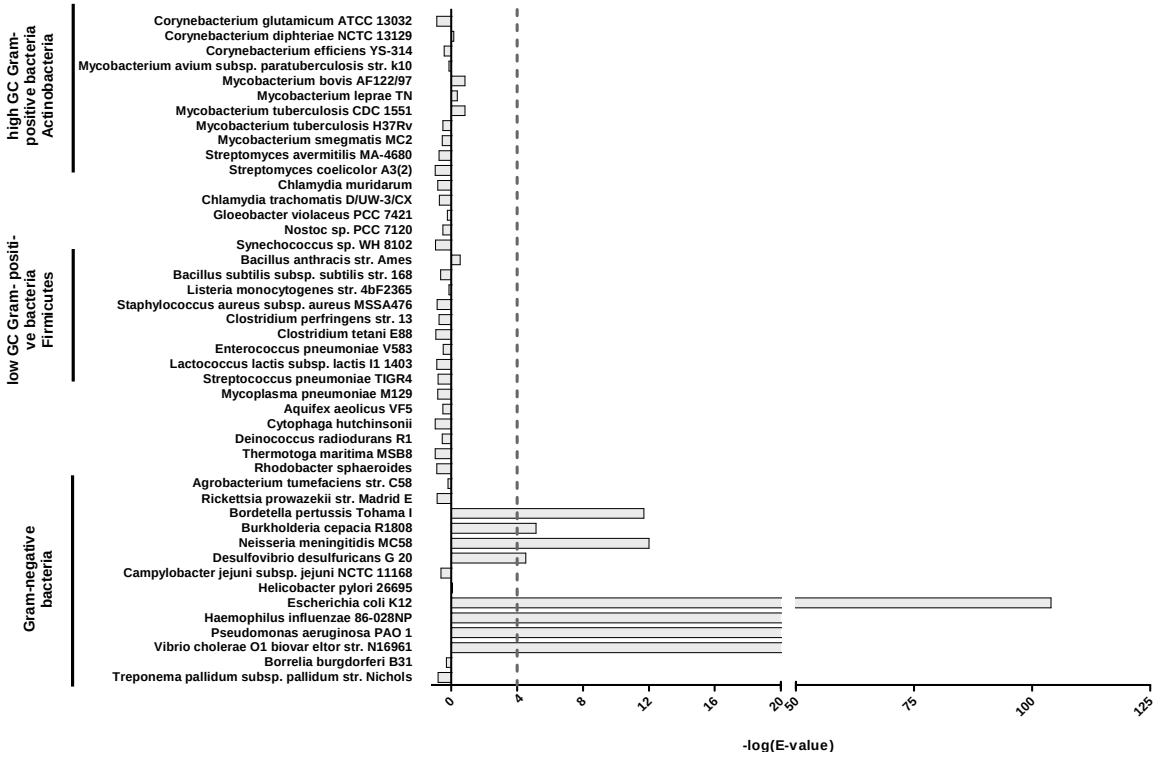


Figure 1. BLASTp search for lipoprotein modifying- and transport proteins. *E. coli* Lnt (c)) and LolA (d)) were used as queries to identify homologues on the National Center for Biotechnology Information BLASTp server. The sequence filtering option was switched off and the expect value was set at 10, the cut-off value set at 10^{-4} (dashed line).

Lipoprotein reporter constructs. In order to investigate the role of the three enzymes Lgt, LspA and Lnt involved in the lipoprotein biosynthesis pathway, subcellular fractionations of *M. smegmatis wt*, Δ*lgt*, Δ*lspA* and Δ*lnt* have been performed according to (Rezwan *et al.*, 2007b). Four different lipoproteins (Table 1) derived from *M. tuberculosis* H37Rv have been chosen for examination and heterogeneous expression in *M. smegmatis wt*- and knock-out strains: Mpt83, which has been localized in the cell wall before by electron microscopy (Vosloo *et al.*, 1997) and by fluorescence cytometry (Harboe *et al.*, 1998), LppX, which is supposed to localize in the cell wall (Mawuenyega *et al.*, 2005; Sulzenbacher *et al.*, 2006), LprN, which has been detected in the cell wall fraction (Mawuenyega *et al.*, 2005) and PstSI which is supposed to localize in the cytoplasmic membrane because of its assumed participation in phosphate uptake and transport across the membrane (Peirs *et al.*, 2005). Due to the fact, that no Lol-homologues have been found as indicated by bioinformatic analyses above, it can be hypothesized that a different signal sequence for transport to the cell wall in mycobacteria has to exist. Additionally, it has been observed by aligning putative mycobacterial lipoproteins, that one or two serines at position +2 or +3 after the cysteine of the lipobox are predominant in lipoproteins presumably located in the cell wall (Chapter 1, data not shown). Therefore, several modified Lpp-constructs have been cloned (Table 1): Mpt83 missing 13 amino acids directly after the Cys of the lipobox (Mpt83Δ13AA), Mpt83Δ13AA with two serines at position +2 and +3 (Mpt83Δ13AA+SS). To prove the existance of a signal sequence determining the subcellular localization of mycobacterial lipoproteins within 10 amino acids after the cysteine at position +1, several “hybrid” constructs were generated by site directed mutagenesis. LppX with 10 amino acids derived from LprN (LppX-10AAN) or PstSI (LppX10AASI) and the counterparts LprN-10AAX and PstSI-10AAX, which correspond to LprN containing 10 amino acids derived from LppX and PstSI with 10 amino acids derived from LppX, respectively (Table 1).

Lipoprotein reporter constructs

Lipoprotein constuct	Protein sequence	Expected localization
	+1 +10 +20	
Mpt83	LAG C SS TKPVSQD TSPKPATSPAAPVTTAA	Cell wall
Mpt83Δ13AA	LAG C -- ------- ----PATSPAAPVTTAA	Cytoplasmic membrane
Mpt83Δ13AA+SS	LAG C SS ------- ----PATSPAAPVTTAA	Cell wall
	*** * ** ******* **********	
LppX	LSG C SS PKPDAEE QGVPVSPTASDPALLAE	Cell wall
	::: : :: ::::::: ::::::::::::::::	
LprN	LAG C QF GGLNSLP LPGTAGHGEGAYSVTVE	Cell wall
	°°° ° °° °°°°°°° °°°°°°°°°°°°°°°°	
PstSI	AAG C GS KPPSGSP ETGAGAGTVATTPASSP	Cytoplasmic membrane
	*** : :: ::::::: **********	
LppX-10AA-N	LSG C QF GGLNSLP QGVPVSPTASDPALLAE	Cell wall
	::: * ** ******* ::::::::::::::::	
LprN-10AA-X	LAG C SS PKPDAEE LPGTAGHGEGAYSVTVE	Cell wall
	*** ° °° °°°°°°° **********	
LppX-10AA-SI	LSG C GS KPPSGSP QGVPVSPTASDPALLAE	Cytoplasmic membrane
	°°° * ** ******* °°°°°°°°°°°°°°°°	
PstSI-10AA-X	AAG C SS PKPDAEE ETGAGAGTVATTPASSP	Cell wall

Table 1: Lipoprotein reporter constructs used in this study. The displayed protein sequence includes the lipobox, with the highly conserved cysteine at position +1. * designates amino acids derived from LppX, **:** from LprN, ° from PstSI. Amino acid deletions are designated by -.

All constructs described above have been fused to the 19 kDa-promoter (a constitutive promoter derived from the 19 kDa lipoprotein) and have been provided with an HA-epitope tag and were cloned into pMV261, a shuttle-vector for *E. coli* and mycobacteria (Stover *et al.*, 1991). The generated vectors were transformed into *M. smegmatis wt*, Δ*lgt*, Δ*lspA* and Δ*lnt* strains. The subcellular localization of the lipoprotein constructs has been explored by Western blot upon fractionation of the bacteria according to Rezwan *et al.* (Rezwan *et al.*, 2007b)

Subcellular localization of lipoproteins of *M. tuberculosis* heterologously expressed in *M. smegmatis*. Figure 2 shows the results of the fractionation of *M. smegmatis wt*, -Δ*lgt*, -Δ*lspA* and Δ*lnt* strains expressing either Mpt83, LppX, LprN or PstSI, all derived from *M. tuberculosis* H37Rv. In the *wt*-strain, Mpt83 (upper row, panel left) can be found in all fractions (total extract, cell wall and cytoplasmic membrane), indicating that Mpt83 is transported to the cell wall which confirms the results obtained with electron microscopy or fluorescence cytometry (Harboe *et al.*, 1998; Vosloo *et al.*, 1997). The fractionation of the Δ*lgt* strain shows the absence of Mpt83 from the cell wall and the cytoplasmic membrane. Mpt83 is absent in the cell wall fractions of the Δ*lspA* and the Δ*lnt* strains but can be detected in the membrane fractions.

In wildtype *M. smegmatis*, LppX shows the same localization Mpt83, which is consistant with observations in previous studies (Mawuenyega *et al.*, 2005; Sulzenbacher *et al.*, 2006). The lipoprotein is absent in the cell wall- and the membrane fraction of the *lgt*-mutant, whilst it is only missing in the cell wall in Δ*lspA* and Δ*lnt* strain. In the *wt*-strain, double bands are detected in the total lysate and in the membrane fraction. The bands in the Δ*lgt*- and Δ*lspA*-strains have a size which corresponds to the upper band in the *wt*. All bands detected in the Δ*lnt*-strain do have the same height, analogous to the lower bands of the *wt*-strain.

LprN can not be detected in the cell wall fraction of any strain, indicating that it is not transported to the cell wall which is contradictory to the findings of Mawuenyega *et al.* (Mawuenyega *et al.*, 2005). Except in Δ*lgt*, where LprN is absent in the membrane fraction as well, the lipoprotein localizes in the membrane fraction.

PstSI, which is predicted to be located in the cytoplasmic membrane (Peirs *et al.*, 2005) is missing in the cell wall fractions of all strains and additionally is absent in the membrane fraction of the Δ*lgt*-strain. These findings support its presumed localization in the membrane as a part of a phospate uptake-system. In summary it has been shown that LppX and Mpt83 are lipoproteins anchored in the cell wall, while LprN and PstSI are located in the cytoplasmic membrane.

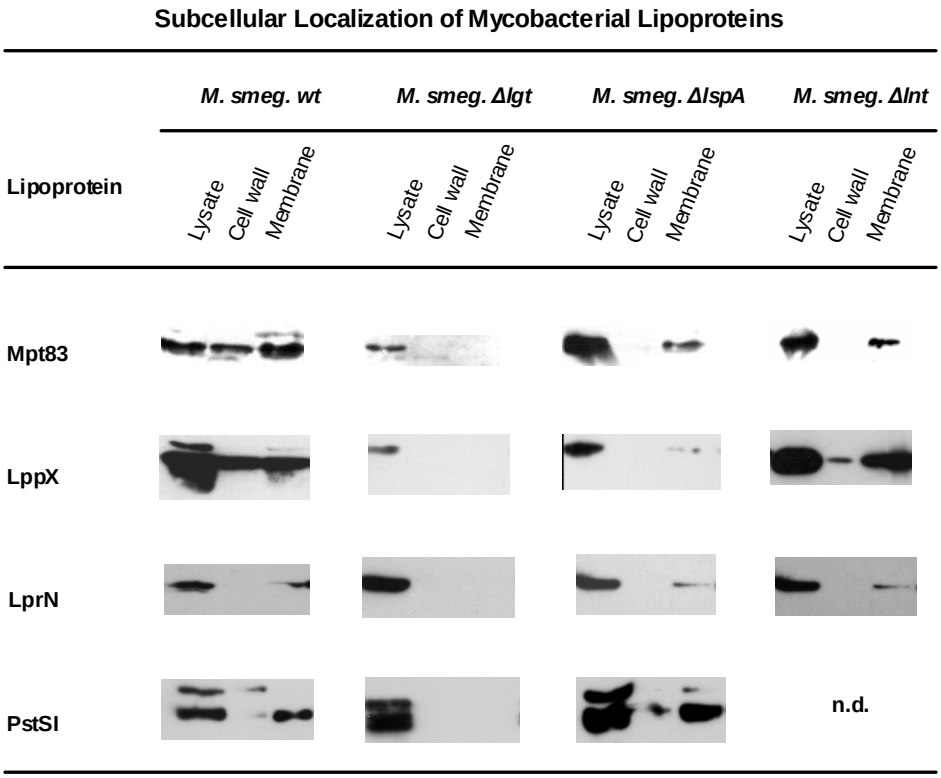


Figure 2: Subcellular localization of *M. tuberculosis*-derived lipoproteins. Western blot developed with α-HA antibody after fractionation of *M. smegmatis wt*, Δ*lgt*, Δ*lspA*, Δ*lnt* strains, transformed with either pMV261-Mpt83, -LppX, -LprN or -PstSI.

Subcellular localization of modified lipoproteins in *M. smegmatis.* We hypothesized, that a signal sequence responsible for the transport to the cell wall of mycobacteria can be found within a couple of amino acids adjacent to the highly conserved cysteine of the lipobox. Therefore recombinant *M. smegmatis* expressing Mpt83Δ13AA and Mpt83Δ13AA+SS were subjected to fractionations. Figure 3 shows that a deletion of 13 amino acids after the cysteine of Mpt83 results in altered localization. Mpt83Δ13AA is absent from the cell wall but found in the membrane fraction. When two serines are re-inserted at positions +2 and +3, the lipoprotein is detected in the cell wall fraction again (Mpt83Δ13AA+SS). These results suggest that these amino acids are part of the signal sequence directing mycobacterial lipoproteins to the cell wall.

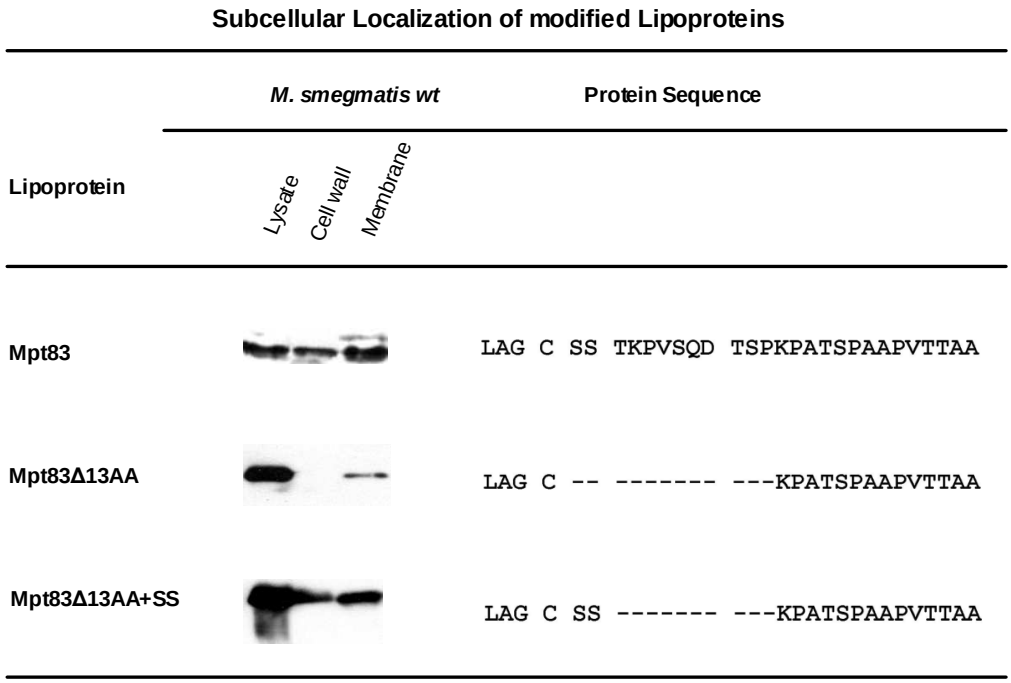


Figure 3: Subcellular localization of modified Mpt83. Western blot developed with α-HA antibody after fractionation of M. smegmatis *wt*-strain transformed with either pMV261-Mpt83, -Mpt83Δ13AA or -Mpt83Δ13AA+SS.

In order to proof whether the cysteine-proximal part of lipoproteins is not only required but also sufficient for cell wall transport, several hybrid constructs were generated. As it has been shown in Figure 2, the native LppX and LprN are located at the cell wall and in the cytoplasmic membrane respectively. The hybrid construct LppX-10AA-N, having replaced 10 amino acids directly after the cysteine by the analogous sequence of LprN, should elucidate whether this sequence of LppX is responsible for cell wall transport. Vice versa, the hybrid construct LprN-10AA-X has been generated. This construct consists of LprN with an exchange of ten amino acids of LppX after the highly conserved cysteine of the lipobox. If these ten amino acids of LppX direct a lipoprotein to the cell wall, the modified LprN is expected to locate at the cell wall.

M. smegmatis wt-strain has been transformed with the vectors pMV261-LppX-10AA-N and pMV261-LprN-10AAX, coding for modified lipoproteins LppX and LprN. Figure 4 shows the results of these fractionations. As controls, the fractionations of unmodified lipoproteins LppX (localization: cell wall) and LprN (localization: cytoplasmic membrane) are displayed. When replacing the subsequent 10 amino acids after the highly conserved cysteine of LppX by the appropriate amino acid sequence of LprN, the lipoprotein is not transported to the cell wall anymore. By contrast, when replacing 10 amino acids of LprN by the according amino acids of LppX, the modified LprN is located in the cell wall.

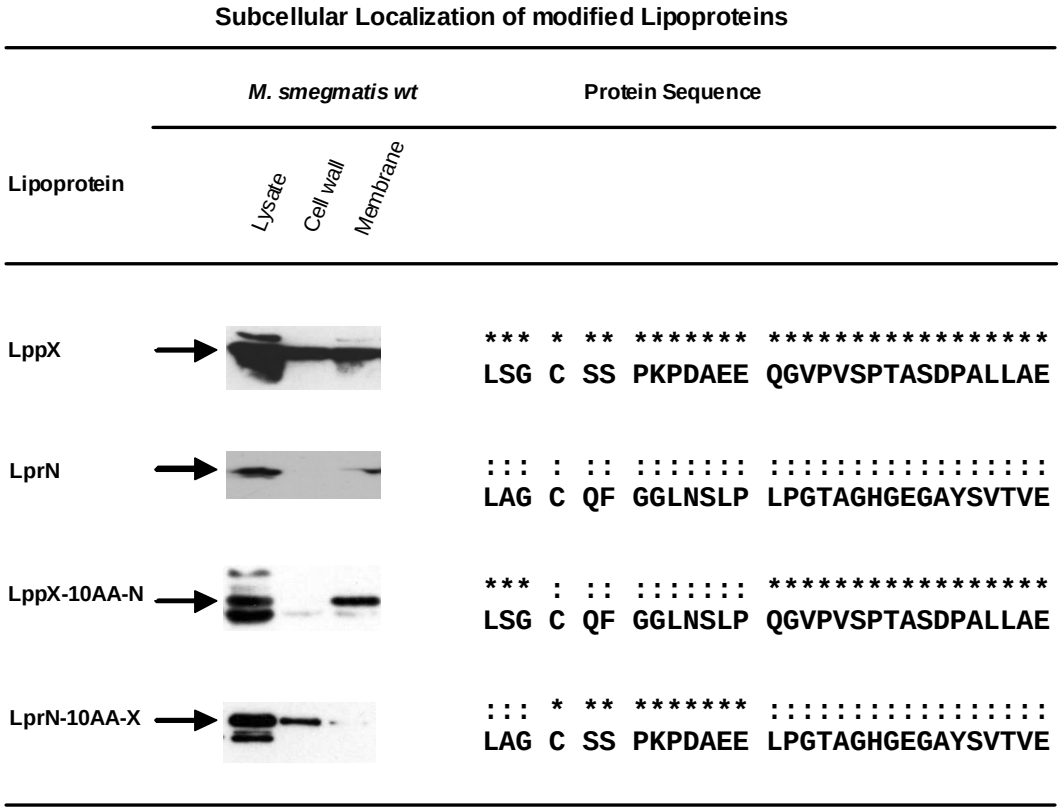


Figure 4: Subcellular localization of modified LppX and LprN. Western blot developed with α-HA antibody after fractionation of *M. smegmatis wt*-strain transformed with either pMV261-LppX, -LprN, -LppX-10AA-N or LprN-10AA-X. Arrows indicate the assumed mature form of the lipoprotein while bands with a lower size are derived from unspecific cleavage.

Similar hybrids to LppX-10AA-N and LprN-10AA-X were constructed for LppX and PstSI, i.e. LppX-10AA-SI and PstSI-10AA-X and transformed into *M. smegmatis wt*-strain. Figure 5 shows the results of these fractionations. LppX and PstSI served as controls (upper panels). As expected, the reporter construct LppX-10AA-SI does not localize in the cell wall anymore. But when replacing the probable signal sequence in PstSI with 10 amino acids of LppX, the hybrid protein localizes in the cell wall.

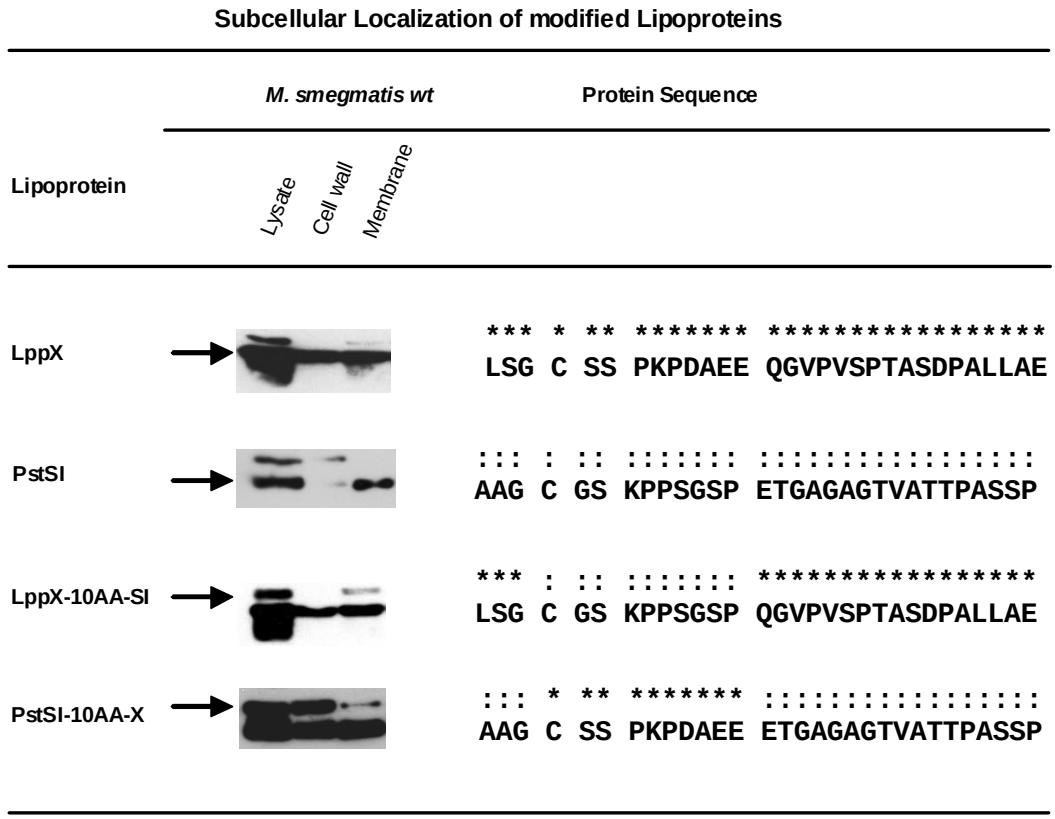


Figure 5: Subcellular localization of modified LppX and LprN. Western blot developed with α-HA antibody after fractionation of *M. smegmatis wt*-strain transformed with either pMV261-LppX, -PstSI, -LppX-10AA-SI or PstSI-10AA-X. Arrows indicate the assumed mature form of the lipoprotein while bands with a lower size are derived from unspecific cleavage.

Discussion and conclusions

In order to investigate a possible signal sequence responsible for cell wall transport of mycobacterial lipoproteins, several *M. tubeculosis* H37Rv reporter constructs have been designed. The lipoproteins under expression of the well known constitutive promoter of the 19 kDa lipoprotein were transformed into different strains of *M. smegmatis*; expression after subcellular fractionation was analyzed by Western blot analyses. As expected, two lipoproteins, Mpt83 and LppX are localized in the cell wall- and the cytoplasmic membrane compartment in *wt*-strain of *M. smegmatis*. This indicates that these two lipoproteins are transported to the cell wall of mycobacteria. PstSI and LprN only have been found in the membrane fraction of *M. smegmatis* which in case of LprN is contradictory to the results of Mawuenyega *et al.* (Mawuenyega *et al.*, 2005). In their study, they found peptides of LprN in the cell wall fraction of *M. tuberculosis* upon two-dimensional liquid chromatography tandem mass spectrometry-analysis. However, this method neither is quantitative, nor did they find

full-length LprN in the cell wall. Peptides of any lipoprotein, localizing in the membrane or in the cell wall, might be found in the cell wall fraction, after their natural degradation by processes called shaving of shedding (Antelmann *et al.*, 2001).

Lipoprotein transport to the outer membrane of *E. coli* is well charactized (Narita & Tokuda, 2006). The first two steps of the lipoprotein biosynthesis are performed by Lgt and LspA. Homologues of these two enzymes have been found by extended protein database search in a wide range of bacteria. Lnt, responsible for the attachment of a third acyl residue to the universally conserved cysteine of the lipobox has been found in Gram-negative but not Gram-positive bacteria. Surprisingly, homologues of Lnt have been found by performing BLASTp in the group of high GC Gram-positive bacteria (Actinobacteria). Even though mycobacteria belong to the group of Gram-positive bacteria, the cellular envelope resembles the envelope of Gram-negative bacteria with an outer membrane. In *E. coli*, the Lol-system is responsible for the release of lipoproteins from the inner membrane and for the transport to the outer membrane. The restrictive conditions for retention of a lipoprotein in the cytoplasmic membrane are the amino acids at position +2 and +3 directly after the modified cysteine of the lipobox. Besides few other combinations, an Asp at position +2 is the major retention signal, meaning that they are not recognized by the Lol-system (Seydel *et al.*, 1999; Terada *et al.*, 2001). By BLASTp search, the five components of the Lol-system solely have been found in some of the examined Gram-negative bacteria. Therefore it is likely that transport to the cell wall of mycobacteria is performed by a completely different mechanism. Likewise, signal sequences responsible for transport to the outer layer of mycobacteria might differ substantially.

In order to identify a signal which directs lipoproteins to the cell wall of mycobacteria, the reporter protein Mpt83Δ13AA has been examined concerning localization in *M. smegmatis wt*-strain. In contrast to the full-length lipoprotein Mpt83, which localizes in the cell wall, the shortened version, missing 13 amino acids directly after the modified cysteine of the lipobox is not transported to the cell wall anymore and remains in the cytoplasmic membrane. This indicates that the construct still is processed as a lipoprotein and therefore is anchored in the membrane with its acyl residues. Secondly, one or a combination of several amino acids within the 13 missing amino acids seems to act as a transport signal to the cell wall. As shown in Table 1 and Figure 3, lipoproteins that are located in the cell wall of mycobacteria do have at least one serine directly after the cysteine of the lipobox. Thus the reporter protein Mpt83Δ13AA+SS has been subjected to subcellular fractionation of *M. smegmatis*. This variant of the lipoprotein LppX lacks 11 amino acids after position +3, positions +1 to +3 are

derived from its native precursor (CSS). Like the native Mpt83, this reporter construct is transported to the cell wall of *M. smegmatis*. These findings indicate that a positive signal sequence for cell wall transport is located within 13 amino acids after the modified cysteine and two serines inserted directly after the lipobox might direct a lipoprotein to the cell wall.

To narrow down the possible signal sequence for transport to the cell wall of mycobacteria, further constructs have been generated and subjected to subcellular fractionation. By site directed mutagenesis, 10 amino acids after the modified cysteine of LppX, which naturally localizes at the cell wall, have been shuffled with 10 amino acids of the membrane-localized lipoproteins LprN and PstSI respectively. In general, unspecific cleavage of these hybrid constructs has been observed, as demonstrated in Figures 4 and 5. Nevertheless, LppX with 10 amino acids derived from one of those lipoproteins localizes in the membrane, indicating that it lacks a sequence which directs it to the cell wall. Vice versa, both lipoproteins that normally are retained in the membrane are directed to the cell wall upon replacing 10 amino acids after the cysteine with the corresponding sequence of LppX.

In summary this study illustrates that the transport of mycobacterial lipoproteins substantially differs from the transport of lipoproteins to the outer membrane of *E. coli*. First of all, no homologues of the Lol-system are present in mycobacteria. Secondly, this study shows that the restrictive conditions for transport to the cell wall of mycobacteria are located within 10 amino acids after the cysteine of the lipobox. Additionally it is more likely that lipoprotein transport in mycobacteria is directed by a “positive” signal sequence (e.g. by two serines at positions +2 and +3), rather than by an avoidance signal like in *E. coli*. More detailed investigations will narrow down a possible transport signal, e.g. by replacing amino acids of membrane-localized lipoproteins at position +2 and +3 by two serines. Knowledge about the cell wall signal sequence of mycobacteria will facilitate the detection of the complete transport system for lipoproteins in mycobacteria.

Experimental section

Bacterial strains and growth conditions. *E. coli* was used for initial cloning and propagation of plasmids. An overnight culture of *E. coli* strain XL-1 blue was inoculated into 1 l of LB medium and cultivated at 37 °C until OD_{600} reached 0.5-0.6. To prepare electrocompetent bacteria, cells were collected by centrifugation at 5000 g at 4 °C for 15 min and washed twice with an equal volume ice-chilled 1 mM HEPES (pH 7.0). The washing procedure was repeated twice with an equal volume of 10% glycerol. Finally, bacteria were resuspended in

5-10 ml of 10% glycerol. The suspension was divided into 40 µl aliquots, frozen in liquid nitrogen and stored at -80 °C until use. For electroporation, 40 µl of competent bacteria were mixed with 2 µl of precipitated ligation reaction or 0.1 µg of supercoiled plasmid DNA (transformation control) respectively and kept on ice for 5 min. Electroporation was performed in 0.1 cm cuvettes with a single pulse (1.8 kV, 2 µF, 200 Ω, Gene Pulser Xcell™, *BioRad*). After a recovery phase of 1 h, the bacteria were selected by plating 100 µl of electroporated bacteria on LB agar plates containing the appropriate antibiotic. Plates were incubated at 37 °C for 14-20 h.

M. smegmatis strains have either been cultivated in LB-Tween or 7H9 medium. After having reached an OD_{600} of 0.6-0.8 bacteria were incubated on ice for 30 min. For preparation of competent cells, the bacteria were collected by centrifugation at 5000 g at 4 °C for 15 min, washed several times with ice-cold glycerol (10% v/v) and finally resuspended in 5 ml glycerol. The suspension was divided into 400 µl aliquots, frozen in liquid nitrogen and stored at -80 °C until use. For electroporation, competent bacteria were thawed on ice and washed once with ice-chilled glycerol (10%). Then, 100 µl competent bacteria were mixed with 1 µg of plasmid DNA and placed on ice for 10 min. Electroporation was performed in 0.4 cm cuvettes with a single pulse (2.5 kV, 25 µF, 1000 Ω, Gene Pulser Xcell™, *BioRad*). Bacteria were immediately resuspended in 1 ml of LB-Tween 80 medium and incubated at 37 °C for 3 h. Electroporated bacteria were selected by plating 100 µl on LB agar or 7H10 plates. Plates were incubated at 37 °C for 3-4 days. When appropriate, gentamicin was added at a concentration of 10 µg ml^{-1}. Strain designations were as follows: *M. smegmatis* Smr5 (*wt*), a strain carrying a non-restrictive *rpsL* mutation conferring streptomycin resistance (Sander *et al.*, 1995), *lgt::aph* = *lgt* knock out mutant (Δ*lgt*); *lspA::hyg* = *lspA* knock out mutant (Δ*lspA*); *lnt::aph* = *lnt* knock-out mutant (Δ*lnt*).

Generation of lipoprotein reporter proteins. All lipoproteins of *M. tuberculosis* were expressed under control of the 19 kDa antigen (*lpqH*) promoter. In general, the recombinant lipoproteins were generated by fusion-PCR; fragment 1 (F1) contains the 19 kDa promoter while fragment 2 (F2) contains the according lipoprotein, including the leader peptide and an HA-epitope tag (hemagglutinin protein) at the carboxy terminus. All reporter proteins have been provided with restritction sites for *Hpa*I and *Eco*RI at both ends. The amplified and fused fragments were cut with *Eco*RI (Fermentas) and cloned into vector pMV261-*gem*, an *E.coli* - mycobacteria shuttle vector with a gentamicin resistance cassette and subsequently transformed into the according strains of *M. smegmatis*. Table 2 gives an overview of the vectors used and generated in this study.

The reporter proteins were generated with the following sets of primer (Table 3): Mpt83: F1 #1, #2; F2 #3, #4; fusion-PCR: #1, #4. LppX: F1 #1, #5; F2 #6, #7; fusion-PCR: #1, #7. LprN: F1: #1, #8; F2 #9, #10; fusion-PCR: #1, #10. PstSI: F1 #1, #11; F2 #12, #13; fusion-PCR: #1, #13. Mpt83Δ13AA+SS (amplified from pMV261-Mpt83Δ13AA): F1 #1, #14; F2 #15, #16; fusion-PCR: #1, #16. LppX-N10AA (amplified from pMV261-LppX): F1 #17, #18; F2 #19 #16; fusion-PCR: #17 #16. LppX-SI10AA (amplified from pMV261-LppX): F1 #17, #20; F2 #21, #16; fusion-PCR: #17, #16. LprN-X10AA (amplified from pMV261-LprN): F1 #17, #22; F2 #23, #16; fusion-PCR: #17, #16. PstSI-X10AA (amplified from pMV261-PstSI): F1: #17, #24; F2: #25, #16; fusion-PCR: #17, #16. If not indicated else, the fragments were amplified from genomic DNA of *M. tuberculosis* strain H37Rv.

Plasmid	Characteristics	Source
pMV261	*E. coli* - mycobacteria shuttle vector, Km^r	(Stover *et al.*, 1991)
pMV261-*gem*	*E. coli* - mycobacteria shuttle vector, Gm^r	this study
pMV261-Mpt83	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-gem, encoding HA-tagged Mpt83 with 19 kDa promoter	this study
pMV261-LppX	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-gem, encoding HA-tagged LppX with 19 kDa promoter	this study
pMV261-LprN	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-gem, encoding HA-tagged LprN with 19 kDa promoter	this study
pMV261-PstSI	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-gem, encoding HA-tagged PstSI with 19 kDa promoter	this study
pMV261-Mpt83-Δ13AA	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-Mpt83, encoding HA-tagged Fus83 with 19 kDa promoter, 13 amino acids deleted after Cys of lipobox	M. Rezwan, internal communication
pMV261-Mpt83-Δ13AA+SS	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-Mpt83, encoding HA-tagged Fus83 with 19 kDa promoter, 13 amino acids deleted after Cys of lipobox and re-insertion of two Ser at positions +2 and +3	this study
pMV261-LppX-10AA-N	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-LppX, encoding HA-tagged LppX with 19 kDa promoter, 10 amino acids substituted by LprN after Cys of lipobox	this study
pMV261-LppX-10AA-SI	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-LppX, encoding HA-tagged LppX with 19 kDa promoter, 10 amino acids substituted by PstSI after Cys of lipobox	this study
pMV261-LprN-10AA-X	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-LprN, encoding HA-tagged LprN with 19 kDa promoter, 10 amino acids substituted by LppX after Cys of lipobox	this study
pMV261-PstSI-10AA-X	*E. coli* - mycobacteria shuttle vector, Gm^r, derivate of pMV261-PstSI, encoding HA-tagged PstSI with 19 kDa promoter, 10 amino acids substituted by LppX after Cys of lipobox	this study

Table 2: Vectors used and generated in this study.

Subcellular fractionation. Subcellular fractionation was performed as described before (Rezwan *et al.*, 2007b) with some minor adaptations. Cells of 1 l culture (7H9) were harvested at 4400 x g and washed once in NaCl solution (0.16 M). Wet weight of the cells was determined and for each gram of bacteria, one ml lysis buffer (0.05 M potassium phosphate, 0.022% (v/v) β-mercaptoethanol, pH 6.5) was added. Cells were broken with a French press (American Instrument Company) two times at 1.1 x 10^8 Pa. The cell lysate was then subjected to a low speed centrifugation at 1000 x g (10 min) to remove unbroken cells. Three to five centrifugation steps (27 000 x g, 45 min) were applied to pellet outer layer material, the supernatant was called SN27.1. All pellets were resuspended with lysis buffer subjected to a second cell lysis step. A single centrifuation at 28 000 x g resulted in pellet P27 (cell wall) and supernatant SN27.2. The supernatants SN27.1 and SN27.2 were pooled, centrifuged at 100 000 x g for 2 hours and thereby seperated into pellet P100 (plasmic membrane) and supernatant SN100 (cytoplasm). All work was done at 4 °C. Subcellular fractions were separated by SDS-PAGE and analyzed by Western blot using anti-HA-antibodies (1:300, Roche). Fractionation was controlled by NADH-oxidase assays (cytoplasmic membrane fraction) and Western blot analyses of cell wall protein MspA (MspA antiserum provided by M. Niederweis, University of Alabama).

Nr.	Sequence	Function	Reporter protein
1	5'- GG**G TTA ACG AAT TC**T ACA TTG CCA CTA CTA CGG TGC -3'	amplification of 19 kDa promoter and signal peptide of *mpt83*	Mpt83 (pMV261Mpt83)
2	5'- CTG AAC GTT GAT CAT CCT GTG CTC CTT TG -3'		
3	5'- CAA AGG AGC ACA GGA TGA TCA ACG TTC AG -3'	amplification of *mpt83* including HA-tag	
4	5'- CC**G TTA ACG AAT TC**T AAG T*GG CGT AGT CGG GGA CGT CGT AGG GGT* ACT GTG CCG GGG GCA TCA		
5	5'- TTT TCC ATC ATT CAT CCT GTG CTC CTT TG -3'	amplification of 19kDa promoter and signal peptide of *lppX*	LppX (pMV261LppX)
6	5'- CAA AGG AGC ACA GGA TGA ATG ATG GAA AA -3'	amplification of *lppX* including HA-tag	
7	5'- CC**G TTA ACG AAT TC**T AAG T*GG CGT AGT CGG GGA CGT CGT AGG GGT* AGT CGA CGT TGA CGG GTT		
8	5'- CCA GAT TCG GTT CAT CCT GTG CTC CTT TG -3'	amplification of 19 kDa promoter and signal peptide of *lprN*	LprN (pMV261LprN)
9	5'- CAA AGG AGC ACA GGA TGA ACC GAA TCT GG -3'	amplification of *lprN* including HA-tag	
10	5'- CC**G TTA ACG AAT TC**T AAG T*GG CGT AGT CGG GGA CGT CGT AGG GGT* ACT GTC CCG ACG CCG TAC CG		
11	5'- CAA ACG AAT TTT CAC CCT GTG CTC CTT TG -3'	amplification of 19 kDa promoter and signal peptide of *pstSI*	PstSI (pMV261PstSI)
12	5' CAA AGG AGC ACA GGG TGA AAA TTC GTT TG -3'	amplification of *pstSI* including HA-tag	
13	5'- CC**G TTA ACG AAT TC**T AAG T*GG CGT AGT CGG GGA CGT CGT AGG GGT* AGC TGG AAA TCG TCG CGA		
14	5'- GGT CGC CGG GCT CGA ACA ACC CGC TAA GAA CGC A -3'	amplification of 19 kDa promoter and signal peptide of *mpt83* Δ13AA+SS	Mpt83Δ13AA+SS (pMV261Mpt83Δ13AA+SS)
15	5'- TTA GCG GGT TGT TCG AGC CCG GCG ACC AGC CCG -3'	amplification of *mpt83* Δ13AA+SS including HA-tag	
16	5'- CC**G TTA ACG AAT TC**T AA*G TGG CGT AGT CGG* -3'		
17	5'- GG**G TTA ACG AAT TC**T ACA TTG -3'	amplification of 19 kDa promoter and signal peptide of *lppX* with 10 amino acids of *lprN*	LppX-N10AA (pMV261LppX-N10AA)
18	5'- CAG CGA GTT CAG CCC GCC AAA CTG ACA TCC TGA TAG CCA CAA C -3'		
19	5'- GGC GGG CTG AAC TCG CTG CCG CTG GTG TTC CCG TGA GCC C -3'	amplification of *lppX* with 10 amino acids of *lprN* including HA-tag	
20	5'- CGA ACC GCT CGG TGG TTT CGA GCC ACA TCC TGA TAG CCA CAA C -3'	amplification of 19 kDa promoter and signal peptide of *lppX* with 10 amino acids of *pstSI*	LppX-SI10AA (pMV261LppX-SI10AA)
21	5'- AAA CCA CCG AGC GGT TCG CCT GAA GGT GTT CCC GTG AGC CC -3'	amplification of *lppX* with 10 amino acids of *pstSI* inclucing HA-tag	
22	5'- CTC GGC ATC AGG TTT CGG CGA AGA ACA GCC GGC AAG CAG CG -3'	amplification of 19 kDa promoter and signal peptide of *lprN* with 10 amino acids of *lppX*	LprN-X10AA (pMV261LprN-X10AA)
23	5'- CCG AAA CCT GAT GCC GAG GAA CAG CCT GGC ACC GCC GGG -3'	amplification of *lprN* with 10 amino acids of *lppX*	
24	5'- CTC GGC ATC AGG TTT CGG CGA AGA ACA GCC CGC CGC TGC TA -3'	amplification of 19 kDa promoter and signal peptide of *pstSI* with 10 amino acids of *lppX*	PstSI-X10AA (pMV261PstSI-X10AA)
25	5'- CCG AAA CCT GAT GCC GAG GAA CAG ACG GGC GCC GGC GCC -3'	amplification of *pstSI* with 10 amino acids of *lppX*	

Table 3: Primer used in this study. **GTT'AAC¦G"AATC**: restriction sites ('': HpaI, **"**: EcoRI), *GG CGT AGT CGG GGA CGT CGT AGG GGT*: HA-tag.

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Identification of Apolipoprotein *N*-Acyltransferase (Lnt) in Mycobacteria

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J. Biol. Chem. (2009), 284(40), 27146-27156

Abstract

Lipoproteins of Gram-negative and Gram-positive bacteria carry a thioether-bound diacylglycerol but differ by a fatty acid amide bound to the α-amino group of the universally conserved cysteine. In *Escherichia coli* the N-terminal acylation is catalyzed by the *N*-acyltransferase Lnt. Using *E. coli* Lnt as a query in a BLASTp search, we identified putative *lnt* genes also in Gram-positive mycobacteria. The *Mycobacterium tuberculosis* lipoprotein LppX, heterologously expressed in *Mycobacterium smegmatis*, was *N*-acylated at the N-terminal cysteine, whereas LppX expressed in a *M. smegmatis lnt*::*aph* knock-out mutant was accessible for N-terminal sequencing. Western blot analyses of a truncated and tagged form of LppX indicated a smaller size of about 0.3 kDa in the *lnt*::*aph* mutant compared with the parental strain. Matrix-assisted laser desorption ionization time-of-flight/time-of-flight analyses of a trypsin digest of LppX proved the presence of the diacylglycerol modification in both strains, the parental strain and *lnt*::*aph* mutant. *N*-Acylation was found exclusively in the *M. smegmatis* parental strain. Complementation of the *lnt*::*aph* mutant with *M. tuberculosis ppm1* restored *N*-acylation. The substrate for *N*-acylation is a C16 fatty acid, whereas the two fatty acids of the diacylglycerol residue were identified as C16 and C19:0 fatty acid, the latter most likely tuberculostearic acid. We demonstrate that mycobacterial lipoproteins are triacylated. For the first time to our knowledge, we identify Lnt activity in Gram-positive bacteria and assigned the responsible genes. In *M. smegmatis* and *M. tuberculosis* the open reading frames are annotated as MSMEG_3860 and *M. tuberculosis ppm1*, respectively.

INTRODUCTION

Proteins of various organisms are modified in numerous ways, one of them is lipidation. Lipid modification of proteins is common in eucaryal and bacterial organisms and can involve myristoyl, palmitoyl, and isoprenyl polymers of various lengths or aminoglycan-linked phospholipids (1, 2). Lipoprotein modifications investigated here are restricted to bacteria. The lipoprotein biosynthesis pathway is a major virulence factor in *Mycobacterium tuberculosis*, the causative agent of human tuberculosis. Every year 1.6 million people fall prey to tuberculosis and one-third of the population of the world are infected. Thus, tuberculosis is responsible for 2.5% of deaths in the world, which is the highest rate claimed by a single infectious agent. An *M. tuberculosis* knock-out mutant deficient in lipoprotein signal peptidase *lspA* showed reduced multiplication in bone marrow-derived macrophages, complete absence of lung pathology and a 1000-fold reduced number of colony forming units in a mouse model of infection (3, 4). Likewise, lipoprotein synthesis contributes to virulence of other Gram positive pathogens, Listeria, Staphylococci, and Streptococci (5).

Bacterial lipoproteins are a functionally diverse class of lipidated proteins involved in cell wall synthesis, nutrient uptake, adhesion, and transmembrane signaling (6) and about 2% of open reading frames encode this kind of proteins (7). Lipidation allows anchoring of these proteins to the cell surface. Lipoproteins are characterized by the presence of a consensus sequence, the “lipobox,” located in the C-terminal part of the leader sequence and consisting of four amino acids (LVI/ASTVI/GAS/C) (7). Precursor lipoproteins are mainly translocated in a Sec-dependent manner across the plasma membrane and modified subsequently on the universally conserved, essential cysteine residue located in the lipobox motif. The modifications taking place after translocation are consecutively mediated by three enzymes: 1) formation of a thioether linkage between the conserved cysteine residue and a diacylglycerol catalyzed by phosphatidylglycerol:pre-prolipoprotein diacylglycerol transferase (Lgt), 2) cleavage of the N-terminal signal peptide by the prolipoprotein signal peptidase/signal peptidase II (LspA), and 3) in the case of Gram-negative bacteria, aminoacylation of the N-terminal cysteine residue by phospholipid: apolipoprotein *N*-acyltransferase (Lnt) (6–8). In *Escherichia coli*, most of the mature triacylated lipoproteins are finally transported across the periplasm by the LolABCDE transport system (9). Homologues of the Lol-transport system are absent in Mycobacteria. Although lipoprotein modifying enzymes act sequentially, Lgt-independent LspA-mediated signal sequence cleavage has recently been demonstrated in *Listeria monocytogenes* (10). Although Lgt and LspA are universally present in both Gram-positive and Gram-negative bacteria, Lnt has been

reported to be restricted to Gram-negative bacteria (11), although some indications for *N*-acylation in *Bacillus subtilis* and *Staphylococcus aureus* were reported (12–15). Mycobacterial lipoproteins are immunodominant antigens (16) and several manipulate innate immune mechanisms and antigen presenting cells (17). It is known that mycobacterial lipoproteins, *e.g.* the 19-kDa lipoprotein, activate Toll-like receptor 2 (TLR2) and co-receptors TLR1, which recognize triacylated peptides, but also TLR6, which recognize diacylated peptides (18, 19). However, the lipid linkage of mycobacterial lipoproteins has not been determined. In this study, we show that Lnt activity is more widely distributed than previously assumed. We demonstrate apolipoprotein *N*-acyltransferase activity in a Gram-positive *Mycobacterium* and give complete structural information about the lipid modification of mycobacterial lipoproteins. Hereby, the functionality of Lnt homologues in actinomycetes is revealed (5). We show that mycobacterial lipoproteins are triacylated and carry mycobacteria-specific fatty acids.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Mycobacterium smegmatis was grown on Middlebrook 7H10 agar supplemented with oleic acid albumin dextrose (OADC, Difco) or LB agar. Tween 80 (0.05%) was added to liquid broth to avoid clumping; when appropriate, antibiotics were added at the following concentrations: 50 μg ml^{-1} kanamycin, 100 μg ml^{-1} streptomycin, and 25μg ml^{-1} hygromycin. Strain designations were as follows: *M. smegmatis* SmR5 (20), a derivative of *M. smegmatis mc*2 (21) carrying a non-restrictive *rpsL* mutation conferring streptomycin resistance (=parental strain); *lnt*::*aph* = *lnt* knock-out mutant; *lnt*::*aph-lntMs* = *M. smegmatis lnt*::*aph* transformed with complementing vector pMV361-hyg-lntMs; *lnt*::*aph-ppm1Tb* = *M. smegmatis lnt*::*aph* transformed with complementing vector pMV361-hyg-ppm1Tb.

Complementation of Conditional E. coli lnt Mutant PAP8508— LntMs was amplified by PCR and cloned into the EcoRI/BamHI sites of pUC18 resulting in pUC18-lntMs. Plasmids pUC18- lntMs323W, pUC18-lntMs477Y, and pUC18-lntMs323W/ 477Y were generated by standard mutagenesis PCR techniques. The *E. coli* conditional *lnt* mutant PAP8508 and its parental strain PAP105 (a generous gift of N. Buddelmeijer) were used for complementation analysis (22). Strains were plated on LB agar supplemented with 1 mM isopropyl 1-thio-β-D-galactopyranoside, 100μg/ml ampicillin, and either 0.4% (w/v) glucose or 0.2% (w/v) arabinose.

Disruption of lnt in M. smegmatis—A 3.8-kbp genomic fragment of *M. smegmatis* from position 3,929,396 to 3,933,223 spanning the entire *lntMs* gene was PCR amplified and cloned into pGem-T Easy (Promega) to result in pGem-T Easy-lntMs. For functional inactivation of *lntMs*, a 1.04-kbp SfiI/EcoRV fragment was replaced with a 1.4-kbp SnaBI/HpaI kanamycin resistance cassette from pUC4K (GE Healthcare) subcloned in pMCS5-Kan.[2] Subsequently a 4.6-kbp PvuII fragment containing the inactivated *lntMs* allele (*lntMs*::*aph*) was inserted into the EcoRV site of ptrpA1-rpsL (20) to result in ptrpA1- rpsL-lntMs::aph. The *lntMs*::*aph* allele was substituted for *lntMs* in the *M. smegmatis* chromosome as described previously (23) and confirmed by Southern blot analyses with a 0.2-kbp SmaI/NcoI *lntMs* upstream probe.

For complementation with *M. smegmatis lnt*, a 4.3-kbp PvuII fragment from pGem-T Easy-lntMs comprising the entire *lntMs* gene was cloned into the HpaI site of plasmid pMV361-hyg (24) to result in pMV361-hyg-lntMs. For complementation with *M. tuberculosis ppm1* a 6.3-kbp fragment from *M. tuberculosis* genomic position 2,306,187 to 2,312,526 spanning the entire *ppm1* gene was cloned into pGem-T Easy to result in pGem-T Easy-ppm1Tb and subsequently subcloned as a 6.3- kbp EcoRI fragment into the HpaI site of plasmid pMV361-hyg (24) to result in pMV361-hyg-ppm1Tb. Complementation was confirmed by Southern blot analyses with a 0.2-kbp SmaI/NcoI *lntMs* upstream probe and a 0.2-kbp KpnI/HindIII *ppm1Tb* upstream probe.

Construction of Expression Vector pMV261-Gm-FusLppX— Plasmid pMV261-Gm, a derivative of pMV261, is a shuttle vector replicating in *E. coli* as well as in mycobacteria (25). *M. tuberculosis* LppX was amplified by PCR from genomic DNA and fused to the *M. tuberculosis* 19-kDa promoter. Two sequences encoding a hemagglutinin and a His_6 epitope were fused to the 3' part of the gene to facilitate subsequent purification and detection on Western blot and the insert was cloned into the EcoRI site to result in pMV261-Gm-FusLppX.

Preparation of Cell Extracts and Western Blot Analysis—Bacteria from 2-liter cultures were harvested, resuspended in phosphate- buffered saline containing Complete EDTA-free tablets (Roche) to inhibit protein degradation, and subjected to two French press cycles (American Instrument Co.) at 2 x 10^8 pascal. Extracts were treated with 2% sodium *N*-lauroylsarcosine for 1 h at room temperature and subsequently incubated at 4 °C overnight. Soluble and insoluble fractions were separated by centrifugation at 30,000 x *g* for 1 h at 4 °C. Extracts corresponding to 1–5 μg of total protein were separated by SDSPAGE (12%) and analyzed by Western blot. Antiserum against the HA^3 epitope (Roche) was diluted 1:300.

Fast Protein Liquid Chromatography Protein Purification— The soluble fraction of cell extracts was diluted with buffer containing 20 mM NaH_2PO_4, 0.5 M NaCl to 1% sodium *N*-lauroylsarcosine and loaded on a HisTrapTM HP column (GE Healthcare) equilibrated with buffer containing 20 mM NaH_2PO_4, 0.5 M NaCl, 0.2% sodium *N*-lauroylsarcosine, and 20 mM imidazole. Proteins were eluted with 0.125– 0.5 M imidazole.

Thrombin Cleavage of RecLppX—Purified RecLppX was dialyzed against phosphate-buffered saline, pH 7.4, at 4 °C because imidazole can lower thrombin activity (26). About 0.1–1 μg of RecLppX was digested with 4–30 NIH units of thrombin from bovine plasma (Sigma) for 16 h at 37 °C with continuous shaking (25 rpm). The reaction was stopped through incubation at 95 °C for 5 min.

MALDI-TOF/TOF—Purified lipoprotein (100–200 pmol) were prepared and analyzed according to Ujihara *et al.* (27). After tryptic digestion samples were resuspended in 5 μl of 0.1% trifluoroacetic acid, 66% acetonitrile. 1.2 μl were loaded onto the target and covered with 1 μl of matrix (α-cyano-4-hydroxycinnamic acid (Bruker Daltonics), 5 mg/ml in 0.1% trifluoroacetic acid, 50% acetonitrile). Mass spectra were recorded on an Ultraflex II MALDI-TOF/TOF instrument with smartbeam laser upgrade (Bruker, Germany). The frequencytripled Nd:YAG laser using a structured-focus profile (smartbeam, Bruker Daltonics) was set to a repetition rate of 100 Hz and the ion acceleration voltage was 29.5 kV. The mass measurements were performed in the positive ion reflector mode.

RESULTS

Mycobacterial Lipoproteins Are Modified at the N-Terminus— We chose the well characterized *M. tuberculosis* lipoprotein LppX (28) as a model substrate for mycobacterial lipoprotein synthesis and generated the expression vector pMV261-Gm-FusLppX. Plasmid pMV261-Gm-FusLppX was transformed into *M. smegmatis* SmR5 (20), *M. smegmatis* Δ*lspA,* and complemented *M. smegmatis* Δ*lspA-lspA.* Whole cell extracts of these strains were subjected to Western blot analysis with anti-HA antibody. We observed bands with an apparent size of 23 kDa in parental and complemented strains and 26 kDa in the Δ*lspA* mutant and to a small amount also in the parental strain. After purification of LppX, the 26-kDa band was also detected to a higher amount in the parental strain. The 23-kDa band corresponds to the predicted mass of mature recombinant LppX-HA-His and 26-kDa band to the predicted mass of the prolipoprotein form of LppX-HA-His. These results indicate LspA-dependent signal peptide cleavage of recombinant LppX-HA- His in *M. smegmatis* and verify its post-translational modification within the lipoprotein synthesis pathway (Fig. 1*a*).

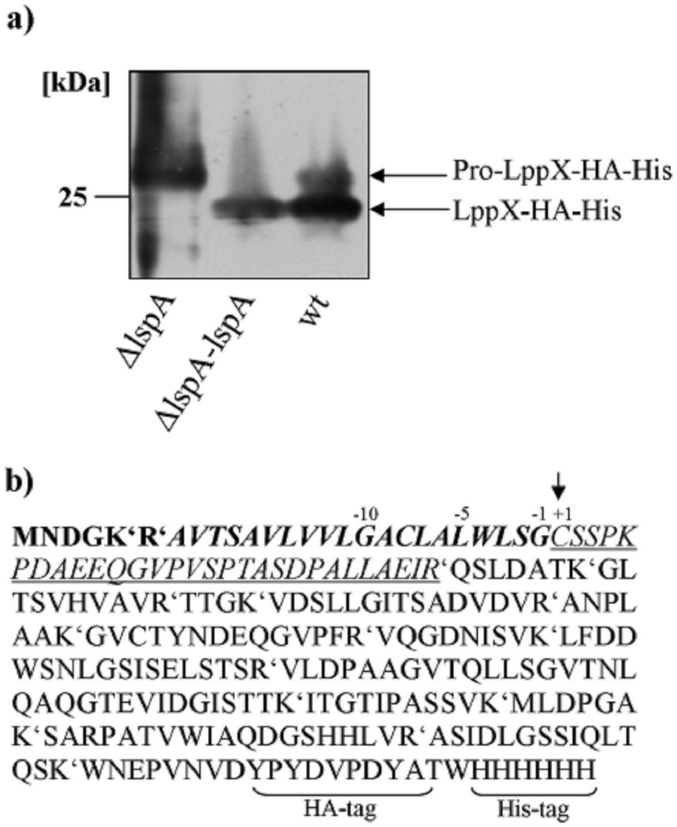

FIGURE 1. Western blot analysis of LppX. a, total lysates of the *M. smegmatis* parental strain, ΔlspA, ΔlspA-lspA expressing LppX with HA and the His_6 epitope analyzed with mouse anti-hemagglutinin monoclonal antibody; b, amino acid sequence of LppX-HA-His. Inverted commas show trypsin cleavage sites. The arrow shows the +1 cysteine modified by Lgt and Lnt. Bold letters indicate the signal sequence cleaved off by LspA reducing the molecular mass by 2.6 kDa. Italic letters indicate the modified tryptic peptide in the ΔlspA mutant, underlined is the N-terminal tryptic peptide found in the *M. smegmatis* parental strain, lnt::aph mutant, and lnt::aph-ppm1Tb.

Purified LppX-HA-His from the *M. smegmatis* parental strain was subjected to protein sequence analysis. Edman degradation of the prolipoprotein revealed a sequence starting at the initial methionine of the signal peptide of LppX (Fig. 1*b*). In contrast, no sequence was obtained from the mature LppX indicating a modification of the N-terminal amino group.

Identification of Putative N-Acyltransferases in Bacterial Genomes—In *E. coli*, *N*-acylation of lipoproteins is conferred by Lnt (29). We performed a BLAST search analysis with *E. coli* Lnt as a query to investigate the distribution of Lnt homologues in the bacterial kingdom and to identify putative homologues in mycobacteria. Lnt homologues are widely distributed in Gram-negative bacteria (α, β, γ, Δ, ε Proteobacteria, Spirochetes, Aquifex, Cytophaga, and Thermotoga), but absent from all classes (Clostridia, Mollicutes, and Bacilli) of low GC Gram-positive bacteria (Firmicutes), although some indications for *N*-acylation in low GC Gram-positive bacteria have been reported (12–15). In contrast, Lnt homologues were identified in all classes of high GC Gram-positive bacteria (Actinobacteria, *e.g.* Streptomyces,

Nocardia, Corynebacteria, and Mycobacteria) (Fig. 2*a*), but Lnt activity of those homologues could not be demonstrated (22). The cell envelope of the phylum Actinobacteria is more complex than the cell envelope of Firmicutes. In *M. tuberculosis* and *M. smegmatis*, Rv2051c (Ppm1) and MSMEG_3860 (Ppm2) have the highest similarity to *E. coli* Lnt. *M. tuberculosis* Rv2051c encodes a two-domain protein, of which the N-terminal part shows similarity to *E. coli* Lnt. The C-terminal part of the protein encodes a polyprenol-monophosphomannose (Ppm) synthase, an enzyme involved in lipomannan and lipoarabinomannan synthesis (30). MSMEG_3860 has been shown to stabilize *M. smegmatis* Ppm1 in the bacterial membrane and therefore has been annotated as Ppm2 (31). MSMEG_3860 will be referred to here as LntMs. Lnt homologues are also present in *Mycobacterium avium* and *Mycobacterium leprae* and are encoded by a separate open reading frame as in *M. smegmatis*. The genomic region surrounding Lnt homologues is conserved in mycobacteria (Fig. 2*b*).

M. smegmatis Lnt Does Not Restore Growth of a Conditional E. coli lnt Mutant—All enzymes of the lipoprotein synthesis pathway are essential in Gram-negative bacteria. We intended to demonstrate mycobacterial Lnt activity by complementation of an *E. coli* conditional *lnt* mutant. Because LntMs is encoded by a separate open reading frame and not fused to a second domain as in *M. tuberculosis*, we chose *M. smegmatis* Lnt (MSMEG_3860) instead of *M. tuberculosis* Ppm1 (Rv2051c) for complementation. LntMs encodes a protein of 654 amino acids with a 25% identity and 40% similarity to *E. coli* and a 63% identity and 73% similarity to the N-terminal part of *M. tuberculosis* Rv2051c. LntMs was cloned into vector pUC18 (Fermentas) to result in pUC18-lntMs and transformed into the conditional *E. coli lnt* mutant PAP8508 (22).

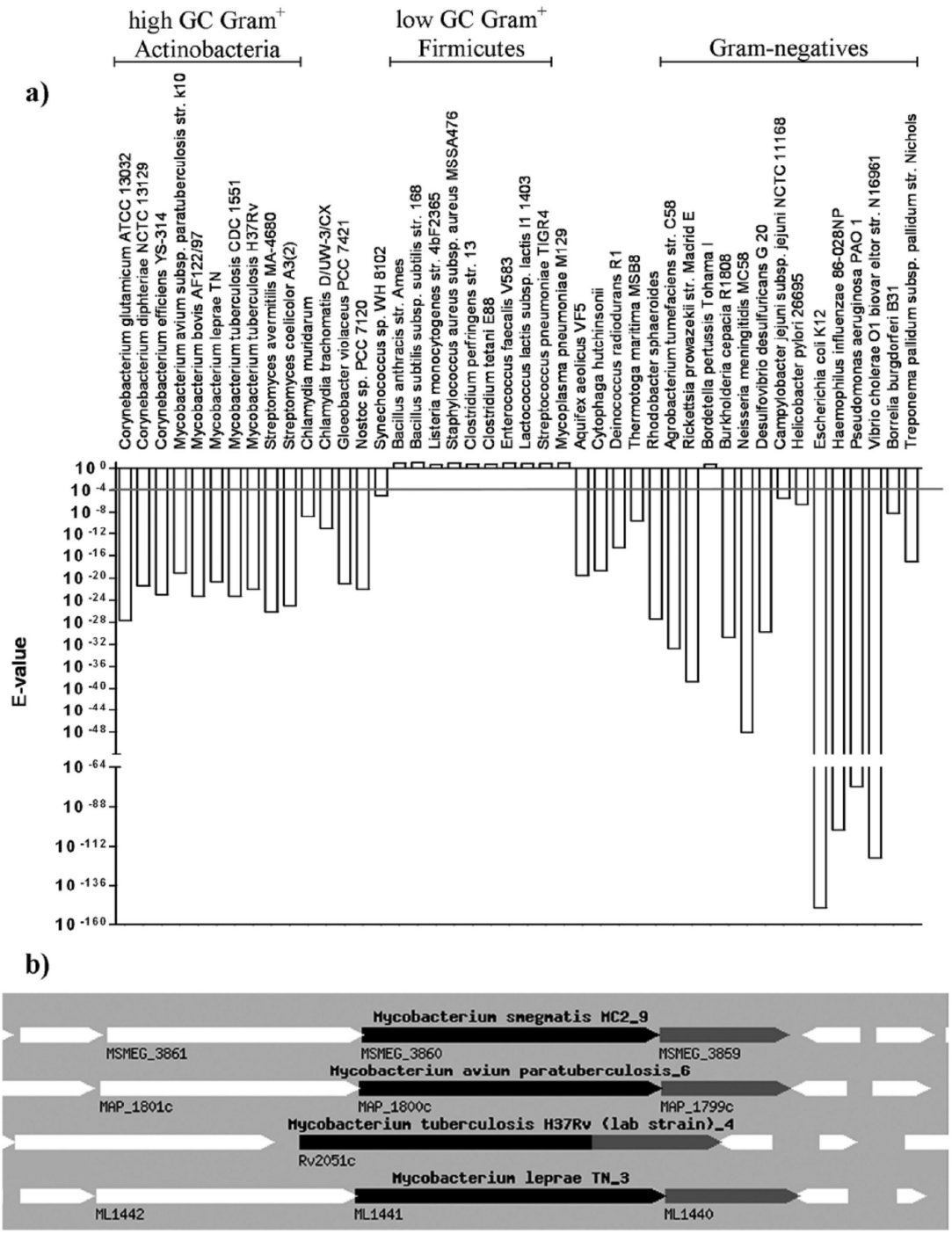

FIGURE 2. **Lnt-BLASTp.** *a*, *E. coli* Lnt was used as a query to identify homologues on the National Center for Biotechnology Information BLASTp server. The sequence filtering option was switched off and the expect value was set at 10, the cut-off value set at 10^{-4}. *b*, a comparison of the genomic region of Lnt (*black*) and Ppm1 (*gray*) homologues in mycobacteria.

However, we could not restore growth of the PAP8508 mutant under restrictive conditions (data not shown). Seven amino acids (Trp-237, Glu-267, Lys- 335, Glu-343, Cys-387, Tyr-388, and Glu-389) are reported to be essential for *E. coli* Lnt function (22). Five of these seven residues are conserved in LntMs, whereas two are altered (LntEc Trp-237 corresponds

to LntMs Glu-323, LntEc Tyr-388 corresponds to LntMs Trp-477). We exploited site-directed mutagenesis to introduce these *E. coli* codons into the *M. smegmatis* sequence of pUC18-lntMs to result in pUC18- lntMs323W, pUC18-lntMs477Y, and pUC18-lntMs323W/477Y. However, transformation of none of these vectors complemented the conditional *E. coli lnt* mutant (data not shown).

Generation and Characterization of M. smegmatis lnt::aph Mutant— Because we were unable to complement an *E. coli lnt* mutant, we decided to investigate Lnt activity directly in mycobacteria by generating a *M. smegmatis lnt* deletion mutant. The deletion mutant was constructed by transformation of *M. smegmatis* SmR5 with the suicide plasmid ptrpA1-rpsLlntMs:: aph using *rpsL* counter-selection strategy (20). The mutant strain resulting from allelic replacement is here referred to as *M. smegmatis lnt*::*aph*. Deletion of *lntMs* was verified by Southern blot analysis using a 5' *lntMs* DNA probe (supplemental Fig. S1). The probe hybridized to a 1.4-kbp fragment of the parental strain and a 6.4-kbp fragment of the *lnt*::*aph* mutant. The difference in size results from the deletion of a BstEII restriction site and insertion of a kanamycin resistance cassette. We cloned two complementation vectors (pMV361- hyg-lntMs and pMV361-hyg-ppm1Tb) expressing *M. smegmatis* Lnt and *M. tuberculosis* Ppm1 under control of their native promoters. Transformation of these plasmids into the *M. smegmatis lnt*::*aph* mutant resulted in strains *M. smegmatis lnt*::*aph-lntMs* and *M. smegmatis lnt*::*aph-ppm1Tb*.

Western blot analysis of extracts from *M. smegmatis lnt*::*aph* expressing LppX-HA-His revealed a molecular mass of the detected protein, which cannot be distinguished from that of LppX-HA-His expressed in the *M. smegmatis* parental strain. However, N-terminal sequencing revealed that LppX-HA-His purified from *M. smegmatis lnt*::*aph* is accessible to Edman degradation (sequence CSSP) indicating that the N-terminal amino group is not still blocked.

LntMs and Ppm1Tb Are Apolipoprotein N-Acyltransferases— Because fatty acids of membrane phospholipids are the substrates for *N*-acylation of lipoproteins in *E. coli* (32–34), its lipoproteins are modified with myristic, palmitic, palmitoleic, oleic, or vaccinic acid (35). Phospholipids in mycobacteria mainly consist of palmitic, palmitoleic, oleic, and tuberculostearic acid (10-methyloctadecanoic acid) (36). Therefore we hypothesized that *N*-acylation of lipoproteins in mycobacteria increase the molecular mass by ~0.3 kDa. To differentiate between lipoproteins with a free or acylated N terminus, we cloned an additional expression vector, RecLppX. It differs from LppX-HA-His by a hemagglutinin epitope followed by a thrombin cleavage site inserted after amino acid Ala (+19) of the mature LppX

(Fig. 1*b*). The thrombin cleavage site LVPRGS was inserted to produce a small N-terminal fragment of 33 residues (about 3.5 kDa) after thrombin cleavage. To ensure that the insertion of a HA epitope and a thrombin site does not abolish recognition of RecLppX as a lipoprotein, we analyzed total lysates of the *M. smegmatis* parental strain, *M. smegmatis* Δ*lspA*, and *M. smegmatis* Δ*lspA*-*lspA* by Western blot (Fig. 3*a*).

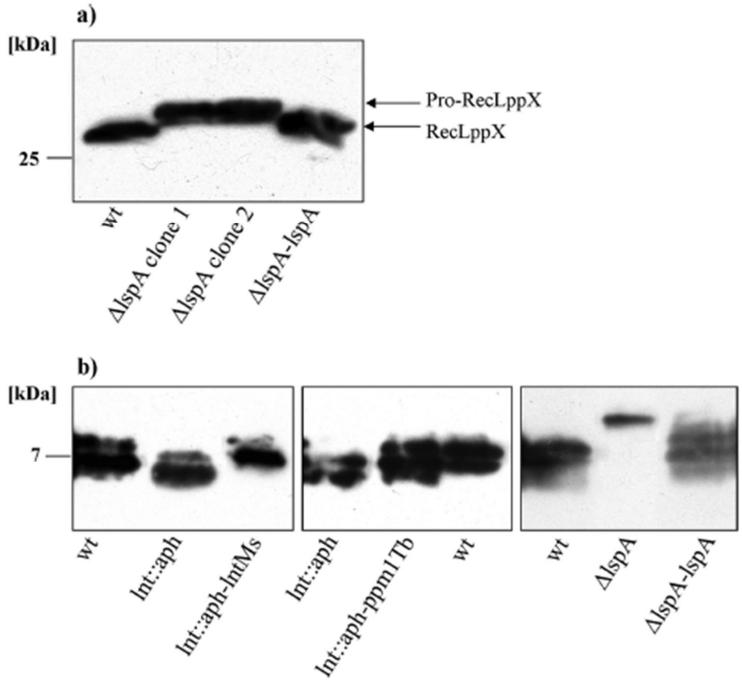

FIGURE 3. Western blot analysis of RecLppX. a, total lysates of *M. smegmatis* wild type (*wt*), ΔlspA, and ΔlspA-lspA expressing RecLppX with hemagglutinin and the His_6 epitope analyzed with mouse anti-hemagglutinin monoclonal antibody. b, RecLppX isolated from indicated strains was digested with thrombin and analyzed with mouse anti-hemagglutinin monoclonal antibody.

Temperature-sensitive *lspA* mutants of *E. coli* and *lspA* knock-out mutants of Gram-positive bacteria accumulate prolipoproteins (37, 38). Immunoblotting of total lysates of the *M. smegmatis* parental strain, Δ*lspA* and Δ*lspA*-*lspA* with antiserum against the HA epitope (Roche) revealed the presence of a 25-kDa band in parental and complemented strains. In contrast, a band with a slightly larger size (approximately 27 kDa, the increase corresponds to the mass of the signal sequence) was observed in the Δ*lspA* strain. This result shows that insertion of a HA epitope and a thrombin cleavage site did not impair the recognition of RecLppX as a lipoprotein. We then investigated thrombin-digested RecLppX for Lnt-dependent modification by Western blot analyses (Fig. 3*b*). In the *lnt*::*aph* mutant we observed a slightly smaller size of the N-terminal part of RecLppX suggesting, that there are

fewer modifications on the protein compared with the parental strain and both complemented strains *lnt*::*aph-lntMs* and *lnt*::*aph-ppm1Tb*. In all strains, we also found a double band of the N-terminal part of RecLppX, indicating partial modification of RecLppX by enzymes other than Lnt or LspA. We also observed a deviation from the calculated size of the N-terminal part of mature RecLppX. The molecular mass was calculated to be 3.5 kDa but in the parental strain we found a band corresponding to a size of about 6 kDa. This difference in size is probably due to an altered migration behavior because of lipid modifications. It can be excluded that these bands at 6 kDa are prolipoprotein forms, still containing the signal peptide, because the N-terminal part of pro-RecLppX from the Δ*lspA* mutant is running at about 8.5 kDa. These results show that RecLppX is modified by LspA as well as LntMs. Ppm1Tb is sufficient to replace LntMs implicating that similar lipoprotein modifications take place in *M. tuberculosis*. Recombinant *M. tuberculosis* LppX (FusLppX) was heterologously expressed and purified. Tryptic fragments of FusLppX were analyzed by MALDI-TOF/TOF mass spectrometry to characterize modifications taking place on lipoproteins in *M. smegmatis* at the molecular level. Purified mature LppX from parental strain, *lnt*::*aph* mutant, and *lnt*::*aph-ppm1Tb* was prepared for analysis according to Ujihara *et al.* (27). For identification of the modifications of the universally conserved cysteine, the structure of the N-terminal tryptic peptide was determined. Experimentally found *m*/*z* values are summarized and compared with calculated *m*/*z* values in Table 1.

TABLE 1
Comparison of m/z values of LppX N-terminal tryptic peptides found in the different mutants
CSS...EIR corresponds to the N-terminal tryptic peptide of LppX upon cleavage of the signal peptide by LspA (Fig. 1b). Mass differences to the corresponding unmodified peptide (upper row) due to modifications are given in paren-theses. Observed modifications are: diacylglycerol with a C16 fatty acid and tuberculostearic acid (C19:0) (+ 592.54 Da), plus eventually *N*-acyl with C16 fatty acid (+ 238.23 Da, Σ = 830.77 Da).

Peptide	Calculated	Parental strain	*lnt::aph*	*lnt::aph-ppm1Tb*
		m/z		
CSS...EIR	2964.46			
CSS...EIR	3557.01		3557.01	3557.07
+Diacyglycerol (C19, C16)	(+592.54)		(+592.55)	(+592.61)
CSS...EIR	3795.24	3795.42		3795.32
+Diacyglycerol (C19, C16)	(+830.77)	(+830.95)		(+830.86)
+*N*-acyl (C16)				

Trypsin cleavage sites of LppX are given in Fig. 1*b*. The expected monoisotopic molecular mass of the unmodified N-terminal tryptic peptide of LppX is 2963.46 Da. Instead, we found a $[M + H]^+$ signal at *m*/*z* = 3795.42 for the N-terminal tryptic peptide of LppX from the

parental strain and a signal at *m*/*z* = 3557.01 from the *lnt*::*aph* mutant. The N-terminal tryptic peptide from *lnt*::*aph-ppm1Tb* is a mixture and showed signals of *m*/*z* = 3795.32 and 3557.07 (Fig. 4). This indicates that the N-terminal peptide of LppX is modified in an LntMs-dependent manner. To identify the found modification, we calculated diacylglycerol modifications with all theoretical combinations of the four fatty acids found in mycobacterial phospholipids: palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), and tuberculostearic acid (C19:0). The difference in molecular mass between the peptide of the *lnt*::*aph* mutant and the unmodified peptide is 592.55 Da indicating a diacylglycerol modification with ester-linked C19:0 and C16:0 fatty acid. These fatty acids are most likely tuberculostearic acid and palmitic acid. The difference in molecular mass of 238.41 Da between the *lnt*::*aph* mutant (*m*/*z* = 3557.07) and parental strain (*m*/*z* = 3795.42) indicates an additional modification with a C16:0 fatty acid in the parental strain. By thoroughly analyzing the signal at *m*/*z* = 3795.42 in high resolution, we also observed a minor signal (approximately 10%) at *m*/*z* = 3793.35. This indicates the presence of a C16:1 fatty acid in place of the C16:0 fatty acid, but only a small amount (data not shown). In the complemented mutant *lnt*::*aph-ppm1Tb* both forms of the N-terminal tryptic peptide of LppX were found. This finding indicates partial complementation of LntMs by *M. tuberculosis* Ppm1. In the *lnt*::*aph* and the *lnt*::*aph-ppm1Tb* mutant we additionally found an N-terminal tryptic peptide at *m*/*z* = 3739.18 and 3739.32, respectively. But no evidence for an aminoacyl modification was found in the MS/MS fragmentation pattern. In contrast, the release of 183 Da most likely corresponds to a covalent modification of the free N-terminal amino group of LppX in these two strains with 4-(2-aminoethyl)-benzenesulfonyl fluoride, a component of the protease inhibitor mixture that has been used. 4-(2-Aminoethyl)-benzenesulfonyl fluoride is known to modify hydroxylated amino acids and to a lesser extent also free amino groups.

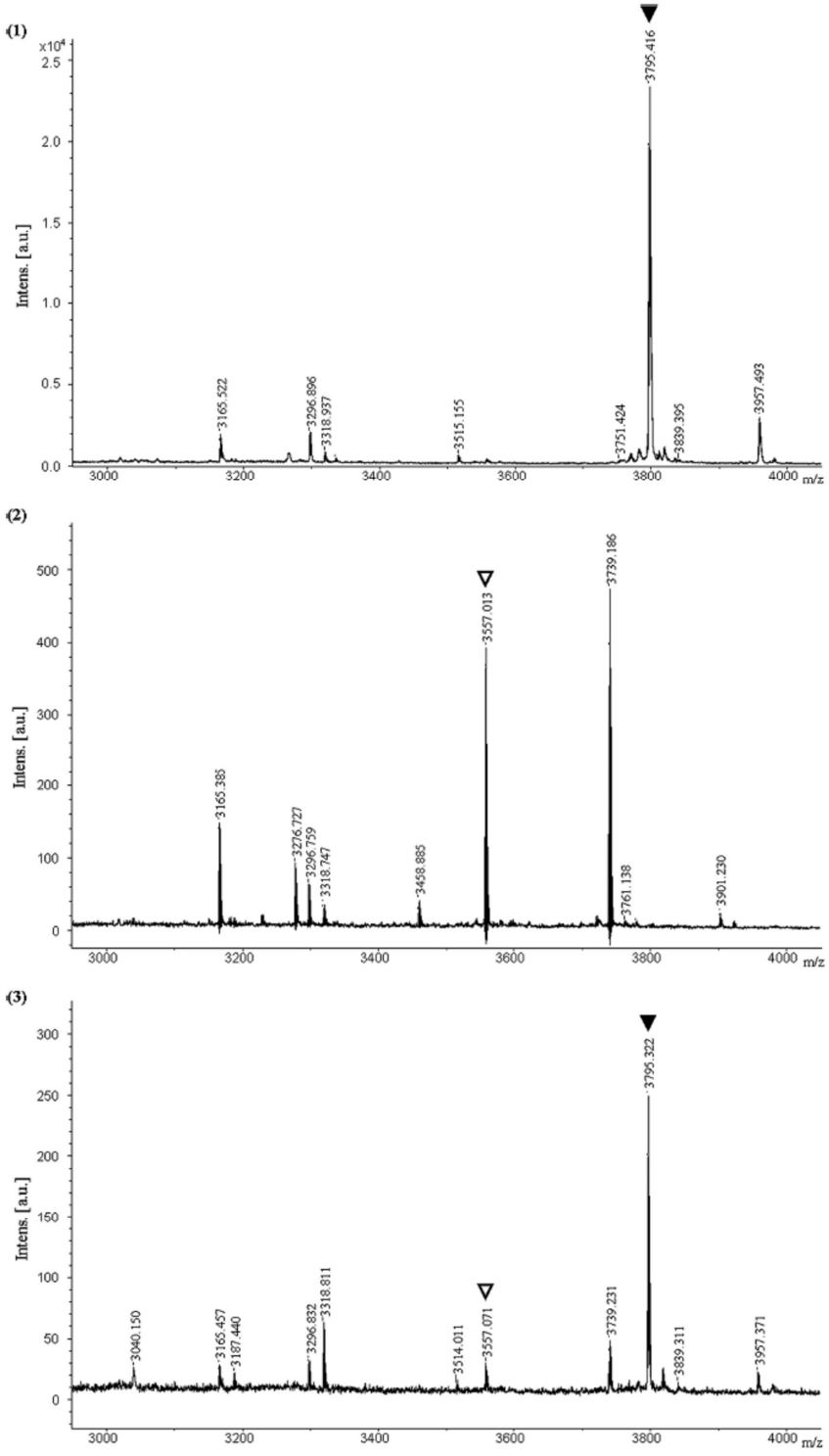

FIGURE 4. **MALDI-TOF analysis of a trypsin digest of purified LppX.** MS analysis of LppX tryptic peptides purified from the *M. smegmatis* parental strain (*1*); *M. smegmatis lnt*::*aph* (*2*), and *M. smegmatis lnt*::*aph-ppm1Tb* (*3*). *Filled triangle*, diacylglycerol plus *N*-acyl modified N-terminal peptide; *open triangle*, diacylglycerol-modified N-terminal peptide.

To obtain information about linkage of the modifications, the structure of the triacylated N-terminal tryptic peptide of LppX (*m*/*z* ± 3795.42) was investigated by MS/MS (Figs. 5 and supplemental S2). A summary of all found eliminations in the three strains are given in Table

2. The ions at *m*/*z* = 3539.59 and 3496.52 correspond to the neutral loss of a C16 (Δ = 255.82 Da) and a C19:0 fatty acid (Δ = 298.89 Da), respectively. The most intense fragment ion at *m*/*z* ± 3169.21 corresponds to the elimination of a diacylthioglycerol carrying both *O*-linked C19:0 and C16 fatty acids (Δ = 626.22 Da). In addition, the release of 370.38 Da from the ion at *m*/*z* = 3539.59 corresponds to the elimination of a C19:0 fatty acid α-thioglycerol ester and the release of 327.31 Da from the ion at *m*/*z* = 3496.52 corresponds to the elimination of a C16 fatty acid α-thioglycerol ester. This fragmentation pattern shows that the +1 cysteine is modified at the sulfhydryl group by a diacylglycerol residue carrying esterbound C16 fatty acid and C19:0 fatty acid. Whether the C19:0 fatty acid is in the S_n1 or S_n2 position cannot be determined. The release of 255.08 Da from the ion at *m*/*z* = 3169.21 indicates the release of a palmitamide derived from an amide-bound C16 fatty acid. The MS/MS fragmentation pattern of the LppX N-terminal tryptic peptide of the *lnt*::*aph* mutant (*m*/*z* = 3557.07) showed only the elimination of the diacylthioglycerol, but no release of palmitamide, as expected (Fig. 5).

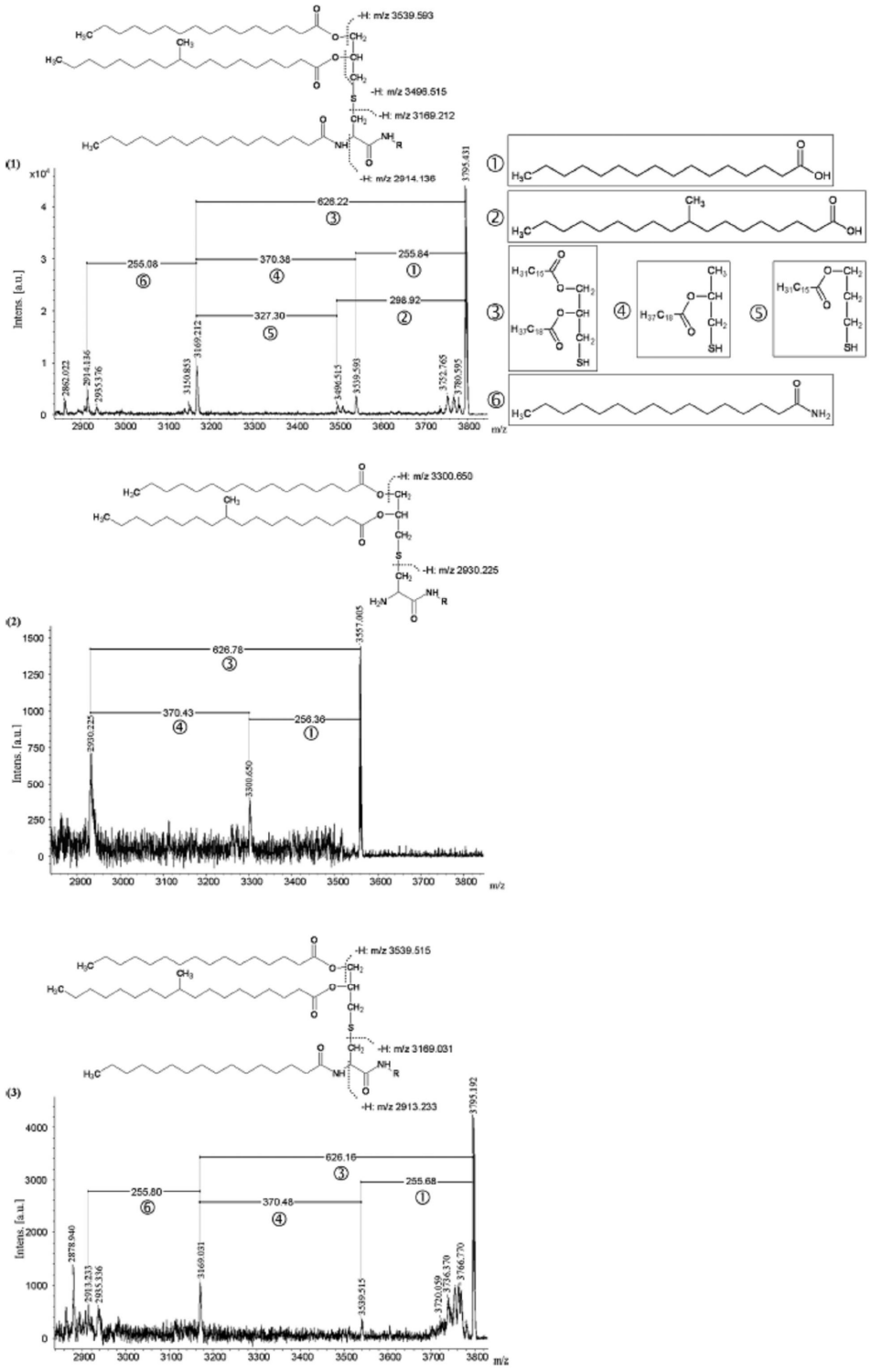

FIGURE 5. **MALDI-TOF-TOF analysis of the N-terminal peptides of LppX.** MS/MS analysis of N-terminal peptides of LppX purified from *M. smegmatis* parental strain (*1*), *M. smegmatis lnt*::*aph* (*2*), and *M. smegmatis lnt*::*aph-ppm1Tb* (*3*). A schematic drawing of the modified +1cysteine with the cleavage sites of each identified *m*/*z* signal is depicted in the *upper part* of each spectrum. The eliminated fragments of LppX modifications are shown on the *right* side of the spectrum (*1*): ①, palmitic acid; ②, tuberculostearic acid; ③, diacylthioglycerol; ④, tuberculostearic acid α-thioglycerol ester; ⑤, palmitic acid α-thioglycerol ester; ⑥, palmitamide. Note that the ester-linked palmitic acid and tuberculostearic acid may be coupled to either positions *Sn*1or *Sn*2. Only one conformation is depicted.

To verify the diacylglycerol modification, we also analyzed tryptic peptides of LppX from the Δ*lspA* mutant by MALDITOF/ TOF mass spectrometry (Fig. 6). Experimentally found *m*/*z* values are summarized and compared with calculated *m*/*z* values in Table 3. Trypsin cleavage sites of pro-LppX are given in Fig. 1*b*. We found three *m*/*z* signals corresponding to the tryptic peptide containing the +1 cysteine. The signal at *m*/*z* = 4887.30 corresponds to the peptide with a disulfide bridge between the two present cysteines (position +8 and +1). The signal at *m*/*z* = 5041.23 corresponds to the peptide with both cysteines being modified by β-mercaptoethanol, a buffer component used for SDS-PAGE. The signal at *m*/*z* = 5558.12 corresponds to the peptide with one cysteine being modified with a β-mercaptoethanol but the other being modified with the diacylglycerol carrying *O*-linked C16 and C19:0 fatty acids also found in the previously analyzed strains (Fig. 6).

TABLE 2
Comparison of experimentall determined eliminations from N-terminal tryptic peptides of LppX in the MALDI-TOF/TOF spectra of the different mutants with theoretically calculated eliminations

Modification	Eliminated fragment	Calculated mass of eliminated fragment[a]	Experimentally determined mass of eliminated fragment		
			Parental strain	*lnt::aph*	*lnt::aph-ppm1Tb*
		Da		*Da*	
O-Linked palmitoyl	Palmitic acid	256.24	255.84	256.36	255.68
N-Linked palmitoyl	Palmitamide	255.26	255.08		255.8
O-Linked tuberculostearyl	Tuberculostearic acid	298.29	298.92		
Diacylglycerol (C16, C19)	Diacylthioglycerol (C16, C19)	626.53	626.22	626.78	626.16
	C16 fatty acid α-thioglycerol ester	328.24	327.3		
	Tuberclostearic α-thioglycerol ester	370.29	370.38	370.43	370.48

[a] Calculated masses are monoisotopic masses.

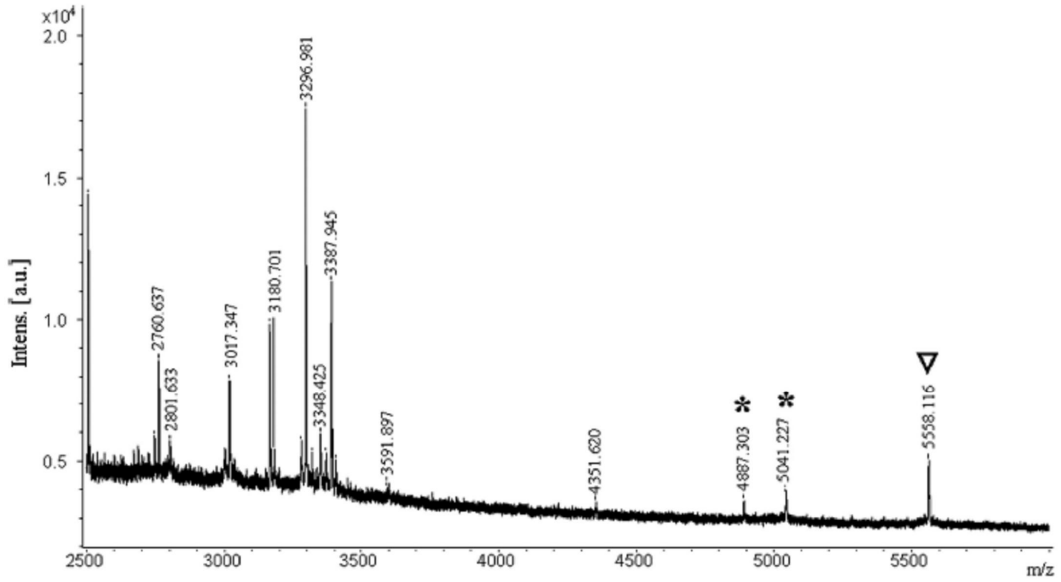

FIGURE 6. MALDI-TOF analysis of peptides resulting from trypsin digestion of LppX from Δ*lspA* mutant. MS analysis of LppX tryptic peptides purified from the *M. smegmatis* Δ*lspA* mutant. Asterisk, N-terminal peptide without fatty acid modifications; open triangle, diacylglycerol modified N-terminal peptide.

TABLE 3
Comparison of *m/z* values of tryptic peptides of pro-LppX containing the +1 cysteine found in the Δ*lspA* mutant
AVT...EIR corresponds to the tryptic peptide of pro-LppX containing the +1 cysteine (Fig 1b). Mass differences to the corresponding unmodified peptide due to modifica-tions are given in parentheses. Observed modifications are: diacylglycerol with C16 and C19 fatty acid (+ 592.54 Da), β-mercaptoethanol (+ 76.00 Da).

Peptide	Calculated	ΔlspA
	m/z	
AVT...EIR with unmodified cysteines	4888.55	
AVT...EIR with S-S	4886.53	4887.3
AVT...EIR	5040.54	5041.23
+ 2X Mercaptoethanol	(+152.00)	(+152.68)
AVT...EIR	5557.09	5558.12
+ 1X Mercaptoethanol	(+668.54)	(+669.57)
+ Diacylglycerol (C19, C16)		

This result shows that LppX purified from the Δ*lspA* mutant is a mixture of pre-pro-LppX and pro-LppX. Taken together the results show that the universally conserved cysteine of *M. tuberculosis* LppX is modified with a thioether-linked diacylglycerol residue carrying an ester-bound C19:0 and an esterbound C16 fatty acid. In addition, it is modified with an amide-linked third C16 fatty acid. The C19:0 fatty acid corresponds most likely to the mycobacterial specific tuberculostearic acid. It is also proved that LntMs is an *N*-

acyltransferase and *M. tuberculosis* Ppm1 is able to complement the *M. smegmatis lnt*::*aph* mutant and therefore Ppm1 seems to be a bifunctional protein. Within this protein the N-terminal domain presumably exhibits *N*-acyltransferase activity (our data) and the C-terminal domain exhibits mannosyltransferase activity (30).

DISCUSSION

The lipoprotein biosynthesis pathway consisting of the three enzymes Lgt, LspA, and Lnt has been intensively studied in *E. coli* and has been shown to be essential and necessary for transport of lipoproteins to the outer membrane of Gram-negative bacteria (11, 39, 40). In mycobacteria, little is known about synthesis and localization of lipoproteins, only a few lipoproteins are functionally characterized and annotation is mainly based on theoretical considerations instead of experimental evidence. However, consistent with the biosynthetic pathway in *E. coli*, putative *lgt* (*Rv1614*) and *lsp* (*Rv1539*) genes have been identified in the *M. tuberculosis* genome (41). In previous studies (3, 4) we showed that in mycobacteria the lipoprotein pathway is a major virulence factor. For fundamental knowledge and further investigations, we were interested in how lipoproteins are modified in mycobacteria. In the present study, we investigated the lipid moieties of a representative mycobacterial lipoprotein.

We identified Lnt homologues in mycobacteria, corynebacteria, and streptomyces species. In low GC Gram-positive bacteria Lnt homologues are completely absent (Fig. 2), but in 1985 the first indirect detection of *N*-acylation in the Gram-positive *B. subtilis* was published and in *S. aureus* triacylation of lipoprotein SitC was recently reported (14, 15), whereas another lipoprotein (SAOUHSC_02699) was only found to be diacylated (13). The protein responsible for attaching the third fatty acid to lipoproteins in *S. aureus* has not been identified. It may be differentially expressed depending on culture conditions or may have a narrow substrate specificity. In *M. tuberculosis* the Lnt homologue found is annotated as Rv2051c. This open reading frame was originally annotated as a two-domain enzyme with a putative N-terminal Lnt domain and a C-terminal polyprenol monophosphomannose synthase (Ppm1) domain and was characterized as the latter one (30). Although the putative Lnt domain is not needed for Ppm1 activity, on overexpression in *M. smegmatis* it appeared to enhance the mannosyltransferase activity. Interestingly, the two domains of *M. tuberculosis* Ppm1 are encoded by separate, adjacent open reading frames in the genomes of other mycobacteria (Fig. 2*b*).

Previous attempts to complement a conditional *E. coli lnt* mutant with Lnt homologues from other bacterial species corresponding to the order Actinomycetales (*Streptomyces* and *Corynebacterium*) failed (22). Likewise we were unable to complement this *E. coli* strain with a mycobacterial Lnt homologue. Even after exchange of the two essential amino acids differing between *M. smegmatis* and *E. coli*, complementation of the *E. coli lnt* mutant failed. LntMs as *E. coli* Lnt attaches a C16 fatty acid to the free amino group of the universally conserved cysteine. Therefore the failure of complementation is not due to the absence of fatty acid substrates. Rather mycobacterial lipoproteins are modified with a diacylglycerol carrying mycobacterial specific fatty acids. Failure of complementation of PAP8508 therefore is probably due to the fact that LntMs recognizes only lipoproteins modified with a diacylglycerol residue carrying at least one ester-bound mycobacterial specific fatty acid. This implies that LntMs does not recognize lipoproteins modified with diacylglycerol residues carrying only small fatty acids like palmitic or palmitoleic acid. Specificity could be tested in an *in vitro* assay system. Alternatively, the expression level or enzymatic activity of mycobacterial Lnt homologues may not sustain growth of fast growing *E. coli*.

We then investigated LntMs and *M. tuberculosis* Ppm1 activity in a mycobacterial background. As *lntMs* is not an essential gene in mycobacteria, we generated an isogenic *M. smegmatis lnt*::*aph* mutant. After thrombin cleavage, the recombinant lipoprotein (RecLppX) extracted from the *M. smegmatis lnt*::*aph* mutant showed a faster running behavior on SDSPAGE than RecLppX extracted from the parental strain. The size was about 0.3 kDa smaller corresponding to fewer modifications of RecLppX in the *lnt*::*aph* mutant. We also recognized a double band of digested RecLppX in all strains used as well as a discrepancy between the calculated and the apparent molecular mass. The altered running behavior is probably due to the modifications on the small N-terminal fragment and the observed double band indicates partial processing of RecLppX by enzymes other than Lgt, LspA, or Lnt. Glycosylation of RecLppX is one possibility, but information about the structure, function, and biosynthetic pathways of prokaryotic glycoproteins is scarce. Glycosylation of *M. tuberculosis* lipoproteins has been confirmed for the 45/47 kDa protein, SodC, and for the *Mycobacterium bovis* MPB83 protein (Rv2873) (42–44). Glycosylation of SodC influences its ultimate subcellular localization and also its proteolytic processing.

By performing MALDI-TOF/TOF analyses of a trypsin digest of purified LppX we unambiguously identified modifications at the universally conserved cysteine. After trypsin cleavage of LppX, the N-terminal peptide from the parental strain has a mass of 3794.41 Da instead of 2963.46 Da predicted for the unmodified peptide, whereas the N-terminal peptide

from the isogenic *lnt*::*aph* mutant showed a mass of 3556.01 Da. This strongly indicates that LntMs covalently links a C16 fatty acid (C16:0 or C16:1) to the N-terminus of the peptide. The additional increase by 592 Da corresponds to a diacylglycerol residue with C16 fatty acid and a C19:0 fatty acid, which corresponds most likely to tuberculostearic acid (10-methyloctadecanoic acid), forming a thioether linkage to the sulfhydryl group of the cysteine. Whether the C19:0 fatty acid is in the *S*n1 or *S*n2 position cannot be determined. The same diacylglycerol modification was also found in the Δ*lspA* mutant. The loss of the *N*-acyl modification in the *lnt*::*aph* mutant is complemented by the *M. tuberculosis* homologue Ppm1 suggesting that mature *M. tuberculosis* lipoproteins are *N*-acylated. *N*-Acylation affects the interaction of lipoproteins with innate immune receptors (45). Ppm1 was shown to exhibit polyprenol-monophosphomannose synthetase activity (30) although the major part of the protein has homology to *E. coli* Lnt. Both masses of the N-terminal tryptic peptide (3556.07 and 3794.32 Da) were found in the complemented mutant indicating that not all apolipoprotein was converted to mature lipoprotein by Ppm1. As *M. tuberculosis* has a generation time of about 24 h and *M. smegmatis* only of about 3 h, it is possible that *M. tuberculosis* Ppm1 has a lower enzymatic activity than LntMs. Alternatively, the expression level of *M. tuberculosis* Ppm1 is lower than that of *M. smegmatis* Lnt. The identification of *O*-linked tuberculostearic acid shows that mycobacterial lipoproteins are modified with mycobacterial specific fatty acids and differ from lipoproteins modified in *E. coli*.

In this study we directly show that Gram-positive mycobacteria synthesize triacylated lipoproteins. This is the first time to our knowledge that responsible genes for Lnt activity are assigned in Gram-positive bacteria. LntMs and *M. tuberculosis* Ppm1 are functional homologues of *E. coli* Lnt as they catalyze the transfer of the third acyl moiety to the free α-amino group of the N-terminal amino acid of lipoproteins. Most likely mycobacterial Lnt homologues differ in substrate specificity from *E. coli* Lnt. *N*-Acylation is a prerequisite for transport of *E. coli* lipoproteins to the outer membrane (46). Likewise, *N*-acylation of mycobacterial lipoproteins may be required for transport to the outer most lipid layer of mycobacteria, which according to recent investigations resembles the outer membrane of Gram-negative bacteria (47, 48).

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Addendum

Personal contribution to chapter 3

In this article, the expression of *M. tuberculosis* lipoprotein LppX, heterologously expressed in *M. smegmatis* was exploited with respect to N-terminal modifications at the molecular level. The lipoprotein biosynthesis pathway of the Gram-negative bacterium *E. coli* was intensively studied previously. It was shown that lipoprotein maturation is mediated by the consecutive activity of Lgt, which attaches a diacylglycerol unit to the sulfhydryl group of the universally conserved cysteine, LspA cleaves off the signal peptide and finally Lnt adds an acyl chain to the N-terminal cysteine. As Lnt was not identified in Gram-positive bacteria, triacylation was uniquely attributed to Gram-negative bacteria. By BLASTp search using *E. coli* Lnt as a query, the last enzyme of the lipoprotein biosynthesis pathway was identified in the Gram-positive genus of *Mycobacterium*. By comparison of LppX-expressing wildtype- or *Δlnt* strains of *M. smegmatis* and MALDI-TOF/TOF analysis, it was shown that mycobacterial lipoproteins are triacylated and carry mycobacteria-specific fatty acids.

My contribution as a second-last author to this manuscript was as follows:

- Development of the fusion-PCR and linker-PCR strategy
- Generation of the initial HA-tagged expression vector
- Transformation into *M. smegmatis* wildtype-, *Δlgt*-, *ΔlspA*- and *Δlnt* strains
- Expression control of the LppX-expressing strains of *M. smegmatis*
- Analysis of the lipoprotein properties of HA-tagged LppX by TritonX114-extraction and LspA-dependent signal peptide cleavage by Western blot Bioinformatic analysis
- BLASTp search on a wide spectrum of bacteria to identify homologues of Lnt
- Writing of parts of the paper

Cloning, expression and characterization of Mycobacterium tuberculosis lipoprotein LprF

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Biochem. Biophys. Res. Commun. 391 (2010) 679–684

Abstract

Lipoproteins are well known virulence factors of bacterial pathogens in general and of *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis, in particular. Lipoprotein lipidation between Gram-positive and Gram-negative bacteria differs significantly as these are di- and triacylated, respectively. Little is known about the lipid anchor of mycobacterial lipoproteins. We reported recently that mycobacterial LppX, a lipoprotein involved in synthesis of cell wall components is triacylated, although mycobacteria are classified as GC-rich Gram-positive bacteria. We here exploited the model organism *Mycobacterium smegmatis* for the expression of *Mtb* LprF and characterized N-terminal modifications at the molecular level. LprF is a putative lipoprotein of *Mtb* involved in signalling of potassium-dependent osmotic stress. LprF is extensively modified in a mycobacterium-specific manner by a thioether-linked diacylglyceryl residue with one ester-bound tuberculostearic- and one C16:0 fatty acid and additionally by a third *N*-linked C16:0 fatty acid, and a hexose.

* These authors contributed equally to this work

Introduction

Tuberculosis is a major cause of death around the world, with 9.3 million new cases and 1.8 million deaths occurring in 2007, which is the highest rate claimed by a single bacterial pathogen (WHO TB-factsheet, 2009). The causative agent of the disease is *Mtb*, an acid-fast bacillus that is primarily transmitted via the respiratory route. The reasons for the pathogens extraordinary success are diverse: it is slow-growing which makes antibiotic treatment complicated and lenghty, it has a thick waxy cell wall and therefore is resistant to different kinds of mechanical and chemical stress and it evades the immune system by parasitizing the macrophages of its host.

The high immunogenic potential of *Mtb* is based on its unusual cell envelope which is exceptionally rich in lipids, glycolipids and polysaccharides (Daffe & Draper, 1998). Amongst others, uncommon cell wall components like mycolic acids, mycocerosic acid, phenolthiocerol, lipoarabinomannan (LAM) and arabinogalactan trigger inflammatory host reactions (Daffe & Draper, 1998). On the other hand, *Mtb* is able to subvert the immune response of the host by inhibiting its innate defense by several mechanisms. It prevents inflammasome activation (Master *et al.*, 2008), delays phagosome maturation in macrophages (Russell, 2001) and suppresses MHC class II antigen presentation (Baena & Porcelli, 2009). Suppression of MHC II antigen expression is conferred by several lipoproteins, e.g. the 19 kDa lipoprotein (LpqH) (Noss *et al.*, 2001).

Lipoproteins are a subclass of proteins found in the cell envelope of all bacteria. Lipoproteins are either di- (in case of Gram-positive bacteria) or triacylated (in case of Gram-negative bacteria) on a highly conserved cysteine located at the N-terminus, which is part of the lipobox [LVI][ASTVI][GAS][C] (Rezwan *et al.*, 2007a). By the consecutive action of the three enzymes pre-prolipoprotein diacyl glyceryl transferase (Lgt), prolipoprotein signal peptidase (LspA) and apolipoprotein *N*-acyltransferase (Lnt), lipoproteins are post-translationally modified after translocation over the cytoplasmic membrane. Lgt attaches a diacylglyceryl residue to the universally conserved cysteine in the lipobox by thioether-linkage. Then LspA removes the lipoprotein signal peptide and Lnt attaches a third acyl chain to the amino group of the modified cysteine. Despite the fact that *N*-acylation in *Bacillus subtilis* and *Staphylococcus aureus* was reported (Hayashi *et al.*, 1985; Kurokawa *et al.*, 2009; Tawaratsumida *et al.*, 2009), Lnt was found exclusively in Gram-negative bacteria. Even though *Mtb* is deemed to be a Gram-positive bacterium because of its staining properties, it has been shown recently that mycobacteria have a periplasmic-like structure (Zuber *et al.*,

2008). Furthermore it has been shown that mycobacteria express a functional Lnt as at least one lipoprotein, LppX was found to be triacylated (Tschumi *et al.*, 2009).

The functions of lipoproteins are manifold; they may be involved in protein export and folding, in antibiotic resistance, in ABC transporter systems, act as substrate-binding proteins and are involved in cell signalling. Examples of mycobacterial lipoproteins which have been explored are Mpt83, LppX, LpqW and the 19 kDa lipoprotein. Mpt83 is assumed to be an adhesin and it has been shown that RNA encoding Mpt83 induces protective immune responses against *Mtb* infection (Xue *et al.*, 2004). LpqW and LppX have been shown to be key players in synthesis and transport of unique components of the mycobacterial cell envelope. Whilst LppX is involved in translocation of phthiocerol dimycocerosates (DIM) to the outer membrane (Sulzenbacher *et al.*, 2006), LpqW has been shown to be essential in the synthesis of the cell wall components phosphatidyl-*myo*-inositol mannoside (PIM) and LAM (Kovacevic *et al.*, 2006; Marland *et al.*, 2006). The 19 kDa lipoprotein has been described as an adhesin (Diaz-Silvestre *et al.*, 2005). It induces IL-1, IL-2 and TNF-α through TLR2-signalling in macrophages. Overall, lipoproteins are important in host-pathogen interactions and they have a high pathogenic potential which has been proven by disruption of *lspA*, the lipoprotein signal peptidase. An *lspA*-deficient strain of *Mtb* exhibited reduced multiplication in mouse macrophages and reduced number of colony forming units in a mouse model by 3-4 logs (Sander *et al.*, 2004).

The putative lipoproteins LprF and LprJ of *Mtb* recently have been described to interact with the histidine kinase KdpD in a yeast two-hybrid screen (Steyn *et al.*, 2003). Both lipoproteins have been suggested to form ternary complexes with the histidine kinase domain of KdpD which in turn seems to be activated after potassium-dependent sensing of environmental osmotic stress and activates a signal transduction pathway.

Even though advances in the past few years contributed to the knowledge of the function of lipoproteins, there is scarcely known anything about the chemical composition of the lipid modifications. Modern tools like mass spectrometry instead of incorporation of radioactive precursors provide support to understand post-translational modifications by lipidation and glycosylation of lipoproteins. Mycobacterial lipoprotein LppX is the first and only lipoprotein characterized at the molecular level (Tschumi *et al.*, 2009). Investigations in other bacteria indicate that lipids of lipoproteins may differ significantly within one species (Kurokawa *et al.*, 2009; Tawaratsumida *et al.*, 2009). Therefore we extended our studies on mycobacterial lipoproteins.

In this study, we cloned, expressed and purified LprF of *Mtb* in *M. smegmatis*, a non-pathogenic but phylogenetically close relative of *Mtb*. We show for the first time that a putatively membrane-localized lipoprotein of *Mtb* is *N*-acylated and determine the predominant fatty acids by matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS).

Material and Methods

Bacterial strains and growth conditions. Mycobacterium smegmatis strains were cultivated in LB medium or on LB agar. If necessary, gentamicin was added at a final concentration of 5 μg/ml. Strains used in this study were *M. smegmatis* SmR5 (Sander *et al.*, 1995), a strain carrying a non-restrictive *rpsL* mutation conferring streptomycin resistance, and *M. smegmatis lnt::aph* mutant (Tschumi *et al.*, 2009).

Expression of recombinant lipoprotein LprF. LprF of *Mtb* was expressed under control of the 19 kDa antigen (*lpqH*) promoter. The recombinant protein was generated by fusion PCR; fragment 1 contains the sequence of the 19 kDa promoter, fragment 2 contains the functional domain of the LprF lipoprotein including the leader peptide and the sequence of a C-terminal thrombin cleavage site and a HA-epitope tag (hemagglutinin protein), followed by a 6x His-epitope tag. Fragment 1 was amplified with primers 1a (5'-GG**GTTAAC** GAATTCTACATTG-3', bold letters indicate sites of restriction enzymes *HpaI* and *Eco*RI) and 1b (5'-*CTTGTGAGATCAAGCCATTCAT*CCTGTGCTCCT-3', italic letters indicate the linker for *lprF*) from plasmid pMV261-Gm-FusLppX (Tschumi *et al.*, 2009). Fragment 2 was amplified with primers 2a (5'-AGGAGCACAGG*ATGAATGGCTTGATCTCACAAG*-3', italic letters indicate the linker for *lprF*) and 2b (5'-CC**GTTAAC**GAATTCTAG TGGTGGTGGTGGTGGTGAGTGGCGTAGTCGGGGACGTCGTAGGGGTAACTACCA CGTGGAACTAGTCCCGCCGGGTTCGG-3', bold letters indicate sites of restriction enzymes *HpaI* and *Eco*RI, underlined letters indicate thrombin cleavage site, HA- and His-tag) from *Mtb* genomic DNA. Fusion PCR was performed with primers 1a and 2b. The recombinant *lprF* gene was cloned into pMV261-Gm (a shuttle vector replicating in *E. coli* as well as in mycobacteria, derived from pMV261) using the *Eco*RI-restriction sites. The resulting plasmid pMV261-Gm-*lprF* was transformed into *M. smegmatis* SmR5 and *M. smegmatis lnt::aph*.

Preparation of cell extracts and Western blot analysis. Bacteria transformed with pMV261-Gm-*lprF* were cultured in 2 l LB medium for 3 days at 37 °C. The cultures were harvested (4400 rpm, 1 h) and resuspended in PBS containing Complete EDTA-free tablets (Roche). Cells were lysed by three French press cycles (American Instrument Company) at 1.1 x 10^6 Pa. Extracts were treated with 2% sodium *N*-lauroylsarcosine (SLS) for 1 h at room temperature, and for 16 h at 4 °C thereafter. Soluble and insoluble fractions were separated by centrifugation at 30000 g for 1 h at 4 °C. Extracts corresponding to 1-5 µg of total protein were separated by a 12.5% SDS-PAGE gel and subsequently analyzed by Western blot using anti-HA-antibodies (1:300, Roche).

Protein fingerprinting. Proteins were digested with trypsin and dissolved in 25 µl 0.1% formic acid. Samples were desalted by using a Ziptip C18 column, mixed 1:1 with matrix solution (5 mg/ml α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) in 0.1% trifluoroacetic acid, 50% acetonitrile) and spotted onto the target.

FPLC protein purification. The soluble fraction of cell extracts expressing epitope-tagged proteins was mixed with dilution buffer (20 mM NaH_2PO_4, 0.5 NaCl, 1% SLS, pH 7.4) and loaded on a HisTrap^{TM} HP column (GE Healthcare) previously equilibrated with equilibration buffer (20 mM Na_2HPO_4, 0.5M NaCl, 0.2% SLS, 20 mM imidazole, pH 7.4). Proteins were eluted applying an imidazole gradient (0.125 - 0.5 M).
As a further purification step, the column flow through from His-tag purification was dialyzed against equilibration buffer (20 mM Tris-hydroxymethyl-aminomethane, 0.1 M NaCl, 0.1 mM EDTA, pH 7.5), loaded onto an anti-HA-affinity matrix (Roche), washed with a buffer containing 20 mM Tris-hydroxymethyl-aminomethane, 0.1 M NaCl, 0.1 mM EDTA, 0.05% (v/v) Tween 20, pH 7.5 and eluted with column regeneration buffer (0.1 M glycine, pH 2.0).

MALDI-TOF/TOF. 100-200 pmol of purified lipoprotein were prepared and analyzed according to Ujihara et *al.* (Ujihara *et al.*, 2008). Lipoproteins were digested with AspN. Extracted peptides were dried and dissolved in 5 µl 0.1% trifluoroacetic acid, 50% acetonitrile. Samples were mixed 1:1 with matrix solution (5 mg/ml α-cyano-4-hydroxy-cinnamic acid (Burker Daltonics) in 0.1% trifluoroacetic acid, 50% acetonitrile) and spotted onto the target. The MALDI-TOF/TOF mass spectra were recorded on an Ultraflex II MALDI-TOF/TOF instrument with smartbeam laser upgrade (Bruker Daltonics). The laser

was set to a repetition rate of 100 Hz and the ion acceleration voltage was 29.5 kV. The mass measurements were performed in the positive ion reflector mode.

Edman degradation. For N-terminal sequencing, proteins were separated by 12.5% SDS-PAGE-gels, blotted onto a PVDF membrane and stained with Coomassie Brilliant Blue (0.03% (w/v) Coomassie Brilliant Blue R-250, 25% (v/v) isopropanol, 10% (v/v) acetic acid) for 5 min. The membrane was destained (10% acetic acid, 35% methanol) and washed once with Aqua B. Braun (Ecotainer®). Visible bands were cut out and analyzed by Edman degradation (Procise 492 cLC protein sequencer, Applied Biosystems), according to the manufacturer's instructions.

Results and Discussion

To analyze the putative lipoprotein LprF from *Mtb* concerning the lipoprotein specific modifications with fatty acids we generated the expression vector pMV261-Gm-*lprF*. Plasmid pMV261-Gm-*lprF* was transformed into *Mycobacterium smegmatis* SmR5 wildtype and an isogenic *lnt*::*aph* mutant strain, lacking a functional apolipoprotein-*N*-acyl-transferase (Lnt). Recombinant LprF from whole cell extracts was purified using the His epitope and subsequently analyzed by Western blot using anti-HA-antibodies. Depending on the kinetics of the enzymes of the lipoprotein biosynthesis pathway [7], the cell extract may contain different forms of the lipoprotein, the pre-prolipoprotein, prolipoprotein, apolipoprotein and the mature lipoprotein. The theoretically calculated molecular masses for LprF are in the range of 26 kDa to 30 kDa. However, the apparent molecular masses estimated from an SDS-PAGE gel may differ significantly. Analysis of the elution fractions from wildtype and *lnt::aph* mutant by Western blot and Coomassie stained SDS-PAGE gels showed bands with an apparent size of 30 and 35 kDa in both strains (data not shown).

Protein analysis. Fingerprint analysis of the 30 and 35 kDa proteins from wildtype and *lnt::aph* mutant confirmed these proteins as LprF from *Mtb*. For characterization and identification of the N-terminal modifications of LprF only the mature lipoprotein is needed. To characterize the 30 and 35 kDa forms of LprF which have been detected after His-tag purification we applied several methods. Edman degradation provides a suitable method to identify the pre-prolipoprotein, prolipoprotein and apolipoprotein. *N*-acylation blocks Edman

degradation. Therefore, MALDI-TOF/TOF MS was used to confirm *N*-acylated LprF forms and to identify modifications at the molecular level.

Edman degradation. The proteins with an apparent size of 35 kDa (Figure 1a) revealed the sequence MNGLI, which is an LprF sequence starting with the initial methionine of the signal peptide (Figure 1c) thereby confirming these forms as pre-pro-LprF or pro-LprF in both strains. The proteins with a size of 30 kDa (Figure 1a) revealed the sequence KKPTT in wildtype and TVVAG in the *lnt::aph* mutant. These determined sequences start at position +3 and at position -5, respectively, in relation to the cysteine (+1) of the lipobox (Figure 1c). These results indicate that the 30 kDa forms of LprF are not the desired LspA-cleaved LprF but LprF forms cleaved by other proteases. The 30 kDa protein form isolated from the *lnt::aph* mutant and starting with the sequence TVVAG contains potentially diacylglycerol modified cysteine and therefore was subjected to MALDI-TOF/TOF MS analysis. The 30 kDa band isolated from the wildtype strain may be a mixture of triacylated LprF and truncated LprF with the N-terminal residues KKPTT. As triacylated lipoprotein is not accessible to Edman degradation due to the blocked N-terminus, the 30 kDa band from wildtype was also subjected to MALDI-TOF/TOF MS.

MALDI-TOF/TOF MS analysis. The Copper chloride stained SDS-PAGE gels of His-tag-purified LprF showed the same bands detected before on Western Blot and Coomassie stained SDS-PAGE gels (Figure 1a). AspN-digested peptides from the 30 kDa recombinant LprF of both strains were analyzed with MALDI-TOF/TOF MS to confirm the N-terminal sequence of mature LprF and to characterize the predominant modifications occurring in *M. smegmatis* lipoprotein LprF at the molecular level. The calculated monoisotopic m/z value for the AspN digested unmodified N-terminal peptide of the LspA cleaved LprF is m/z = 2496.2 (Figure 1c). The fatty acids found in mycobacterial phospholipids are palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1) and tuberculostearic acid (10-methyloctadecanoic acid) (C19:0) (Goren, 1979). Since fatty acids of membrane phospholipids are used for *N*-acylation of lipoproteins in *E. coli* (Jackowski & Rock, 1986; Lai & Wu, 1980), we calculated the theoretical mass of the N-terminal peptide of LprF with all possible combinations of the above mentioned four fatty acids. Lipoproteins sometimes are glycosylated (Sartain & Belisle, 2009) and putative sites for *O*-glycosylation are also present in the N-terminal AspN-fragment of LprF. Therefore we also calculated the mass with several hexose modifications. However, no signals corresponding to the free or acylated N-terminal peptides (with or without

glycosylations) were found in the mass spectrum (data not shown). So, the analyzed 30 kDa proteins of LprF are most likely the non-modified but truncated proteins, as indicated by the results of the Edman degradation.

Analysis of HA-tag purified LprF. Since the 35 kDa LprF clearly was identified as the (pre-) pro-LprF and the 30 kDa forms were not the LspA-processed LprF we assumed that the expected mature forms were not yet isolated. Concentrating again on the Western blots and Coomassie stained SDS-PAGE gels from His-tag purification, we found another distinct band on the Western blots from the column flow throughs. This band had an apparent size of 28 kDa possibly corresponding to the LspA-cleaved LprF (data not shown).
Due to the fact that this protein form did not bind to the HisTrap™ HP column, we used the HA-epitope in the recombinant LprF to purify this protein from the flow through fractions. After purification with the HA-affinity matrix, the proteins with the size of 28 kDa in wildtype and *lnt::aph* mutant were observed on Western blot, Coomassie and Copper chloride stained SDS-PAGE gels as well (Figure 1b).

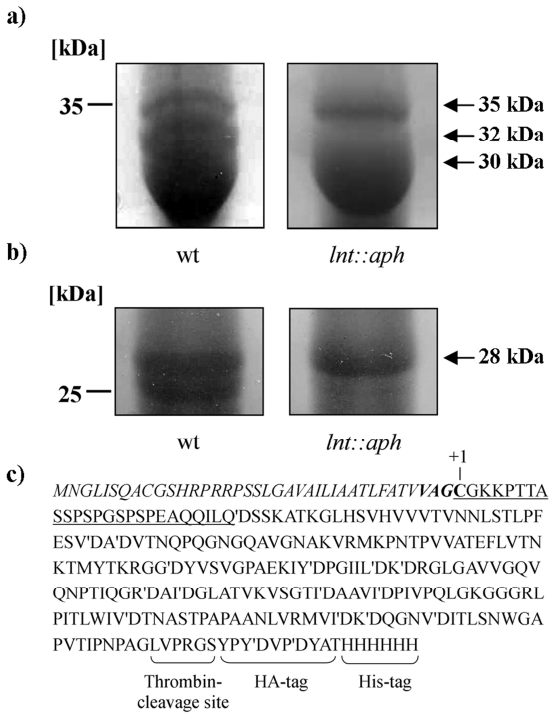

Figure 1: Copper chloride stained SDS-PAGE gels and amino acid sequence of purified LprF
a) HisTrap™-column purified LprF from *M. smegmatis* wildtype and *lnt::aph* mutant. b) HA-affinity matrix purified LprF from *M. smegmatis* wildtype and *lnt::aph* mutant. Proteins at 25 kDa were identified as histone-like protein HupB from *M. smegmatis*. c) Amino acid sequence of recombinant LprF. Italic letters indicate the signal peptide cleaved by LspA. Bold letters indicate the lipobox including the conserved cysteine at position +1 modified by Lgt and Lnt. Inverted commas indicate AspN cleavage sites. The modified N-terminal peptide after AspN digestion found in *M. smegmatis wt* and *lnt::aph* mutant is underlined.

Edman degradation of HA-tag purified LprF. To determine whether these 28 kDa forms from wildtype and *lnt::aph* mutant are the LspA-cleaved LprF or LprF at all, Edman degradation was performed although for the mature lipoprotein from wildtype N-terminal sequencing is expected to be blocked by the modified N-terminus.

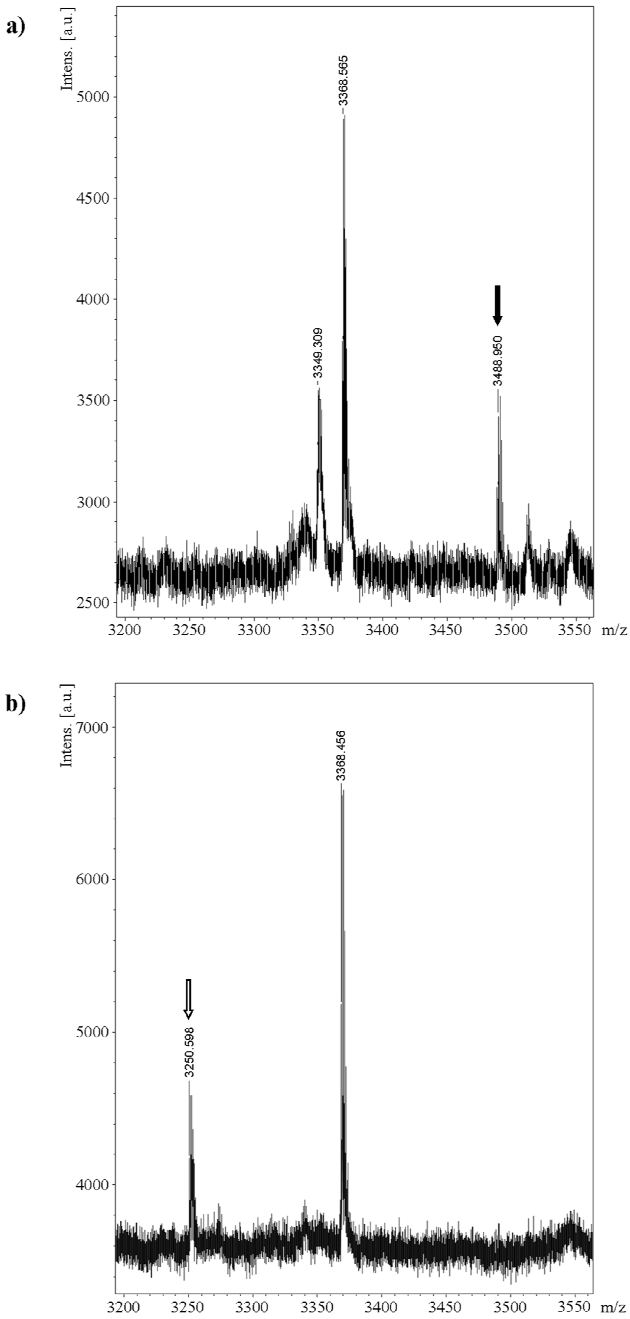

Figure 2: MALDI-TOF MS analysis of AspN digested purified LprF (28 kDa)
MS data of AspN digested LprF peptides from a) *M. smegmatis* wildtype and b) *M. smegmatis lnt::aph* mutant. Black arrow indicates triacylated monoglycosylated N-terminal peptide. Open arrow indicates diacylated monoglycosylated N-terminal peptide. The mass at *m/z* = 3368.5 corresponds to an internal AspN peptide of LprF (residue 201 – 232 of the mature lipoprotein).

The analyses revealed the unique sequence xxKKP in LprF indicating that these proteins start with the cysteine at position +1 (Figure 1c) thereby confirming these proteins as the LspA-cleaved LprF in the wildtype and the mutant. As the resolution of the SDS-PAGE gel is not sufficient to separate triacylated and diacylated lipoproteins, the 28 kDa band from wildtype may be a mixture of both forms.

MALDI-TOF/TOF MS analysis of HA-tag purified LprF. For the identification of the lipoprotein specific modifications at the conserved cysteine the AspN digested peptides from the 28 kDa LprF from wildtype and *lnt::aph* mutant were investigated by MALDI-TOF MS. Instead of the $[M+H]^+$ signal at *m/z* = 2496.2 as calculated for the AspN-digested, unmodified N-terminal peptide we found a signal at *m/z* = 3488.9 for the modified N-terminal peptide of LprF from wildtype (Figure 2a). In the *lnt::aph* mutant we found a signal at *m/z* = 3250.5 (Figure 2b), indicating a smaller size of the N-terminal peptide and thus suggesting an Lnt dependent modification in the wildtype. The difference in molecular mass between the unmodified N-terminal peptide (*m/z* = 2496.2) and the peptide found in the *lnt::aph* mutant (*m/z* = 3250.5) is 754.3 Da indicating a diacylglyceryl modification with ester-linked tuberculostearic acid and C16:0 fatty acid (592.5 Da) and a glycosylation with one hexose (162.2 Da, Σ = 754.7). The difference in molecular mass of 238.4 Da between wildtype (*m/z* = 3488.9) and mutant indicates an additional modification of the N-terminal peptide with a C16:0 fatty acid in the wildtype.

In order to confirm the modifications of the AspN digested N-terminal peptide of LprF from wildtype (*m/z* = 3488.9) and *lnt::aph* mutant (*m/z* = 3250.5), the structure of the N-terminal peptide was analyzed with MALDI-TOF/TOF MS. In wildtype (Figure 3a) the ion at *m/z* = 3326.7 corresponds to the cleavage of a hexose (Δ = 161.2 Da). The ion at *m/z* = 2698.7 is the most intense ion and corresponds to the release of the diacylthioglyceryl carrying both, an *O*-linked tuberculostearic and a C16:0 fatty acid (Δ = 628.0 Da). The release of 257.1 Da from the ion at *m/z* = 3326.7 corresponds to the elimination of a C16:0 fatty acid and the release of 370.9 Da from the ion at *m/z* = 3069.6 corresponds to the elimination of a tuberculostearic acid α-thioglyceryl ester. The difference in molecular mass between the MS signal for the N-terminal peptide from wildtype and *lnt::aph* mutant is 238.4 Da which indicates a third acylation with a C16:0 fatty acid in the wildtype peptide in an Lnt dependent manner.

In the *lnt::aph* mutant (Figure 3b) the ions at *m/z* = 3088.8 and 2461.9 correspond to the release of a hexose (Δ = 161.8) and a diacylthioglyceryl carrying both *O*-linked tuberculostearic and C16:0 fatty acid (Δ = 626.9), respectively. Of note - a loss of the His-epitope in this 28 kDa LprF forms was assumed to be responsible for the failure to extract this

proteins by the His-tag purification. But, the presence of the tag was confirmed by MALDI-TOF/TOF MS analysis (data not shown).

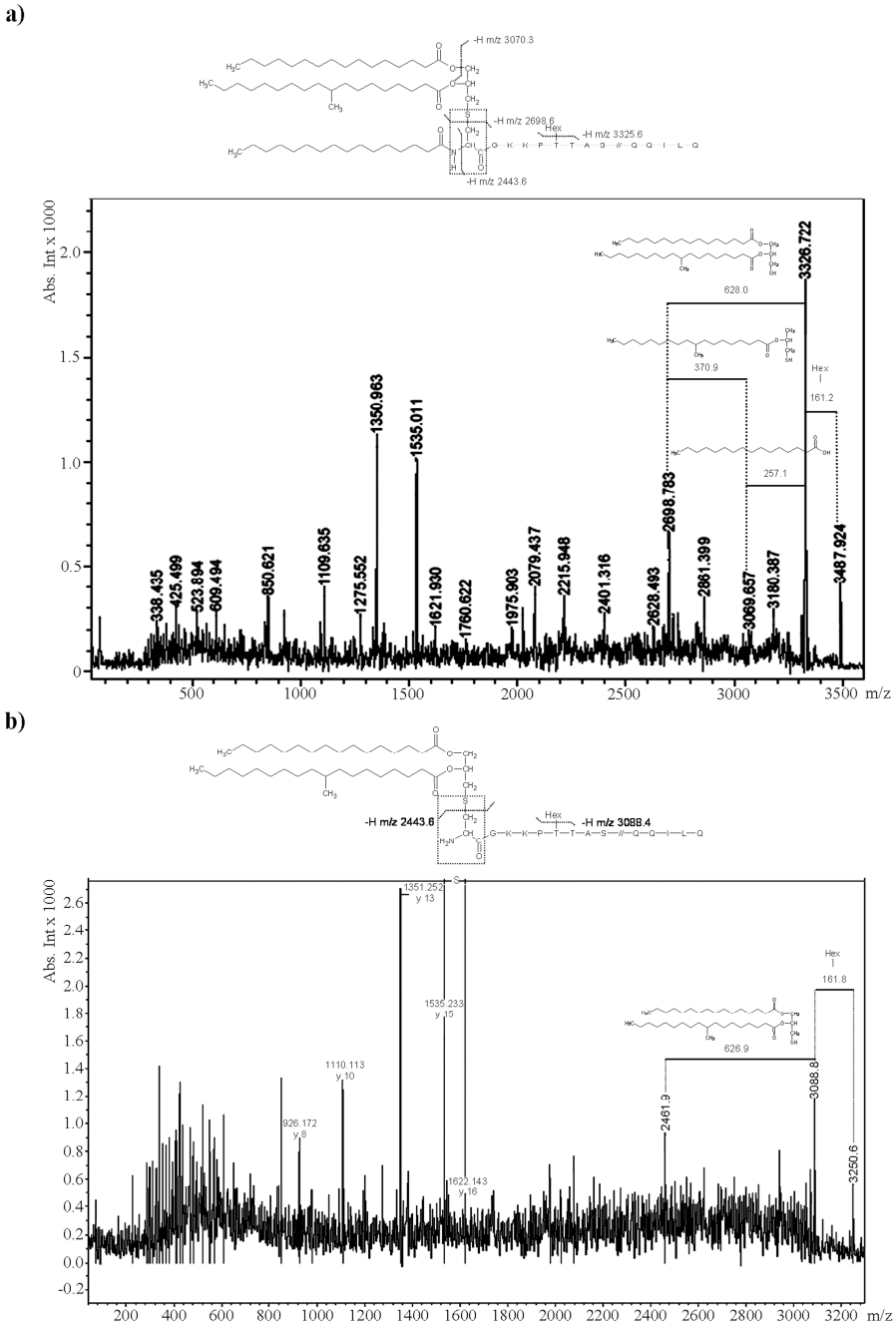

Figure 3: MALDI-TOF/TOF MS analysis of N-terminal peptides of LprF
MS/MS data of the N-terminal peptides of LprF from a) *M. smegmatis* wildtype and b) *M. smegmatis lnt::aph* mutant. Schematic drawings of the structure of the modified N-terminal peptide of LprF are shown in the upper part of each MS/MS spectrum. Dotted frames indicate the modified conserved cysteine at molecular level. Cleavage sites of each identified *m/z* signal are indicated. Eliminated fragments of LprF modifications are depicted in the spectra.

Conclusions

This study shows for the first time the modifications of a putative membrane located lipoprotein of *Mtb* on the molecular level. The LprF of *Mtb* wildtype is a triacylated and glycosylated lipoprotein carrying a thioether linked diacylglyceryl residue with an ester-bound tuberculostearic- and C16:0 fatty acid and a third C16:0 fatty acid most likely at the amino terminal cysteine residue. These results together with the recent analysis of LppX [16] indicate that *N*-acylation seems to be a common motif in mycobacterial lipoproteins. As expected for the *lnt::aph* mutant the cysteine in LprF is modified with the diacylglyceryl residue, but is missing the third acylation (only glycosylated and modified with the diacylglyceryl residue,) thereby confirming lipoprotein acylation by MSMEG_3860 in mycobacteria. Due to the close phylogenetic relation between *M. smegmatis* and *Mtb* the same acylation pattern of LprF in *Mtb* is assumed. Although glycosylation of the N-terminal AspN peptide of LprF was identified, the exact glycosylation site of seven possible sites within the peptide could not be determined.

Fingerprinting and Edman degradation identified several proteins isolated either by His- or HA-affinity purification as LprF. However, the N-terminal lipidated LprF could not be purified by His-tag purification suggesting that the lipidation interferes with His-purification despite the presence of the His-tag which was proved by analysis of the C-terminus (data not shown) or somehow reduces the binding affinity of the protein. Beside the pre-pro-lipoprotein, apolipoprotein and the mature LprF a protein with an apparent size of 32 kDa found only in the wildtype was confirmed by fingerprint as LprF as well, but was not subjected to further analysis in this study. This LprF may result from cleavage by proteases other than LspA.

Acknowledgments

We thank Rene Brunisholz from the Functional Genomics Center Zurich for performing N-terminal sequencing. This work was supported in part by the Swiss National Science Foundation (3100A0-120326) and the European Union (TB-Drug: LSHP-CT-2006-037217).

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Addendum

Personal contribution to chapter 4

In this article, the post-translational modifications of LprF, a lipoprotein derived from *M. tuberculosis*, expressed in *M. smegmatis* was characterized. It was shown for the first time that lipoproteins which are localized in the cytoplasmic membrane are tri-acylated in the same manner as lipoproteins which are localized at the cell wall of mycobacteria. By applying MALDI-TOF/TOF analysis it was shown that LprF is modified by a thioether-linked diacylglycerol residue with one ester-bound C19:0- (tuberculostearic acid) and one C16:0 (palmitic acid) fatty acid and additionally by a third *N*-linked C16:0 fatty acid and a hexose.

My contribution as shared-first author to this manuscript was as follows:

- Design of the study
- Generation of expression vectors
- Data analysis
- Writing of the manuscript

A synthetic mammalian gene circuit reveals antituberculosis compounds

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Proc. Natl. Acad. Sci. USA (2008), 105(29), 9994-9998

Abstract

Synthetic biology provides insight into natural gene-network dynamics and enables assembly of engineered transcription circuitries for production of difficult-to-access therapeutic molecules. In *Mycobacterium tuberculosis* EthR binds to a specific operator (O_{ethR}) thereby repressing *ethA* and preventing EthA-catalyzed conversion of the prodrug ethionamide, which increases the resistance of the pathogen to this last-line-of-defense treatment. We have designed a synthetic mammalian gene circuit that senses the EthR–O_{ethR} interaction in human cells and produces a quantitative reporter gene expression readout. Challenging of the synthetic network with compounds of a rationally designed chemical library revealed 2-phenylethyl-butyrate as a nontoxic substance that abolished EthR's repressor function inside human cells, in mice, and within *M. tuberculosis* where it triggered derepression of *ethA* and increased the sensitivity of this pathogen to ethionamide. The discovery of antituberculosis compounds by using synthetic mammalian gene circuits may establish a new line of defense against multidrug-resistant *M. tuberculosis*.

Introduction

Up to 9 million people contract tuberculosis every year and 50 million people are presently infected with *Mycobacterium tuberculosis* resistant to both first-line drugs isoniazid and rifampicin (1) [World Health Organization (WHO), fact sheet no. 104, March 2007]. Ethionamide, a structural analogue of isoniazid, is currently the last line of defense in the treatment of multidrug-resistant tuberculosis (MDR-TB). During 35 years of its clinical use, ethionamide has fortunately elicited little cross-resistance with isoniazid because both prodrugs have to be activated by different mycobacterial enzymes to develop their antimicrobial activity (2). Ethionamide is activated by the Baeyer–Villiger monooxygenase EthA, which converts the prodrug into an antimycobacterial nicotinamide adenine dinucleotide derivative (3, 4). Because *ethA* is repressed by EthR (5), ethionamide-based tuberculosis therapy is often unsuccessful even when prescribed at high hepatotoxic doses (6). Therefore, compounds preventing EthR from binding to the *ethA* promoter could increase the sensitivity of multidrug-resistant *M. tuberculosis* to ethionamide and make tuberculosis treatment safer, more efficient, and affordable. Crystallography-based structural analysis implied that hexadecylocanoate copurifying with EthR could abolish EthR's operator-binding capacity (7). However, hexadecyloctanoate turned out to be too hydrophobic to confirm this hypothesis in any cell/microbial culture system suggesting that it remains a nontrivial challenge to discover bioavailable EthR-binding compounds. Because *M. tuberculosis* is an intracellular pathogen, EthR inhibitors do not only have to specifically target the bacterial repressor, but also need to reach the cytosol without eliciting any cytotoxic effect. Therefore, integrated screening approaches assessing specificity, bioavailability, and cytotoxicity in a single assay are expected to rapidly reveal valid drug candidates. Although synthetic mammalian gene networks designed so far, including epigenetic toggle switches (8), hysteresis networks (9), time-delay circuits (10), and synthetic ecosystems (11), have resulted in important information on the dynamics of physiologic control systems, the EthR-based gene circuit pioneers a direction with a more practical purpose: providing a generic screening platform to discover drug candidates with the potential to efficiently kill *M. tuberculosis*, the causative agent of one of the most devastating human diseases.

Results

Design of an EthR-Based Synthetic Mammalian Gene Circuit. Structural analysis (7, 12) classifying EthR as a TetR/CamR family repressor suggested the existence of compounds that could modulate the affinity of EthR for its O_{ethR} operator (13, 14). Adopting a synthetic biology approach we have designed a gene network whose topology enabled detection of EthR-binding molecules inside human cells, thereby scoring for noncytotoxic and bioavailable compounds accessing the pathogenic habitat of *M. tuberculosis* (Fig. 1a). The gene circuit consists of a synthetic transactivator, EthR, fused to the VP16 transactivation domain of Herpes simplex (pWW489, P_{SV40}-EthR-VP16-pA), which induces SEAP (human placental secreted alkaline phosphatase) expression in human embryonic kidney cells (HEK-293) after binding to a chimeric promoter containing the EthR-specific operator (O_{ethR}) 5' of a minimal Drosophila heat shock protein 70 promoter ($P_{hsp90min}$; pWW491, $O_{ethR\text{-}Phsp70min}$-SEAP-pA) (8.2 ± 0.8 units/liter; background level of pWW491, 0.4 ± 0.1 units/liter) (Fig. 1a). Cell-permeable EthR-interacting compounds were expected to release EthR-VP16 from $O_{ethRPhsp70min}$, thereby repressing SEAP production to basal levels (Fig. 1a). Interestingly, hexadecyloctanoate (10 mM), identified in crystallography studies to compromise EthR's DNA-binding capacity (7), failed to decrease SEAP expression in HEK-293 cells containing pWW489 and pWW491 (data not shown), which is probably because of its highly lipophilic structure (ClogP = 11.29). Therefore, it remains a nontrivial challenge to discover bioavailable EthR-binding compounds as they must be sufficiently lipophilic to fit into the hydrophobic tunnel of EthR (7) while being hydrophilic enough to reach therapeutic levels in the bloodstream and inside infected cells.

Discovery of Compounds Affecting the DNA-Binding Affinity of EthR. Capitalizing on crystallography data describing EthR's small-molecule binding site as "hydrophobic tunnel-like cavity fitting a lipophilic ligand" (7, 12) and on the observation that repressors are often feedback-controlled by the products of their target gene (15, 16), we have synthesized a library of hydrophilic esters (ClogP < 4), a substance class, which is the main product of EthA-catalyzed Baeyer–Villiger oxidation (Fig. 1b). When HEK-293 populations containing the EthR-based gene circuit (Fig. 1a) were exposed to 0–3.2 mM individual library components, only benzylacetate, 3-phenylpropyl-propionate, 2-phenylethyl- butyrate, and 4-phenyl-2-butanone [a ketone class control (7)] induced a significant decrease in SEAP expression, suggesting that these compounds may trigger the release of EthR from O_{ethR} (Fig. 1c). However, because 3-phenylpropyl-propionate and 4-phenyl-2-butanone were also reducing SEAP levels of HEK-ET-SEAP cells transgenic for constitutive SEAP expression

(Fig. 1c), these substances were considered cytotoxic at EthR-releasing concentrations [SEAP production and viability of HEK-ET-SEAP cells were shown to correlate; supporting information (SI) Fig. S1]. Therefore, only benzylacetate (IC_{50} = 1 mM) and 2-phenylethyl-butyrate (IC_{50} = 0.5 mM) were used for further studies (Fig. 1c).

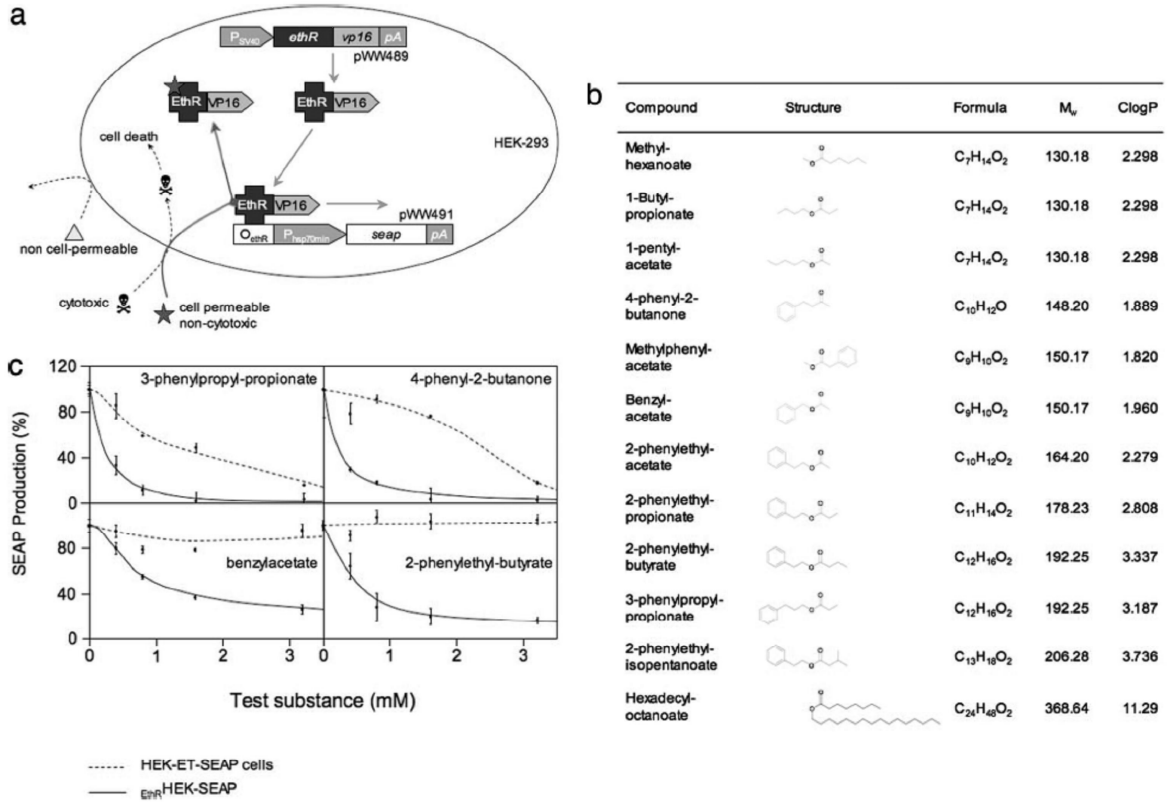

Fig. 1. EthR-based synthetic gene network in mammalian cells. (a) A gene fusion of *ethR* with the Herpes simplex-derived *vp16* transactivation domain is expressed under the control of the simian virus 40 promoter (PSV40, plasmid pWW489) in HEK-293. The chimeric transactivator EthR-VP16 binds to its operator O_{ethR} thereby activating transcription from the minimal Drosophila heat shock 70 promoter ($P_{hsp70min}$), driving expression of human placental secreted alkaline phosphatase (seap, plasmid pWW491). In the presence of a cell-permeable, noncytotoxic inducer, binding of EthR-VP16 to the promoter is inhibited, thereby resulting in transcriptional silence (red lines). Non-cell-permeable or cytotoxic compounds are automatically excluded from the hit list. (b) Compounds selected for testing as potential inducers of EthR. The ClogP value indicates the calculated distribution coefficient between n-octanol and water. (c) Screening of a rationally designed compound library by using the EthR-based gene network. 30,000 HEK-293 containing either the EthR-based gene network (pWW489 and pWW491, EthR-HEK-SEAP cells) or an isogenic constitutive SEAP expression network (pWW35 and pWW37, HEK-ET-SEAP cells) were cultivated for 48 h in the presence of potential inducers before SEAP profiling. SEAP expression was normalized to 100%.

Validation of EthR-Modulating Compounds in Bacteria and in Vitro. When exposing *Escherichia coli* engineered for P_{ethR}-driven GFP expression to benzylacetate and 2-phenylethyl-butyrate concentrations used for the aforementioned experiments with mammalian cells, dose-dependent GFP expression could be observed, indicating that these compounds were also able to trigger release of EthR from O_{ethR} in prokaryotes (Fig. 2 a and b;

see Fig. S2 for expression constructs and experimental setup). Similar to mammalian cells, hexadecyloctanoate was unable to modulate EthR's P_{ethR}-binding affinity in *E. coli* at concentrations up to 10 mM (data not shown). Benzylacetate efficiently released EthR from its operator but did so only at elevated concentrations (10 mM) that are known to be mutagenic and likely incompatible with future therapeutic use (Material Safety Data Sheet, Sigma). We have therefore focused on 2-phenylethyl-butyrate and further characterized the adjustable EthR-O_{ethR} release capacity of this licensed food additive in a cell-free ELISA system (Fig. 2c): Hexahistidine-tagged EthR (EthR-His_6) was allowed to bind to agarose beads containing immobilized O_{ethR} in the presence of increasing concentrations of 2-phenylethyl-butyrate and O_{ethR} interacting EthR-His_6 was quantified after a washing step by using a His_6-specific horseradish peroxidase-coupled antibody and a standard assay system (Fig. 2c). The inverse correlation of 2-phenylethyl-butyrate and O_{ethR}-bound EthR indicates that this compound is able to induce release of EthR from its operator. The specificity of 2-phenylethyl-butyrate's EthR-releasing capacity was confirmed by an identical control experiment with an unrelated repressor–operator interaction (Fig. S3).

2-Phenylethyl-butyrate Modulates EthR Activity in Mice. To evaluate whether the licensed food additive 2-phenylethyl-butyrate [Joint Food and Agriculture Organization (FAO)/WHO Expert Committee on Food Additives, JECFA no. 991], retains its regulating activity in vivo, we stably transfected the EthR-based gene circuit into HEK-293 cells ($_{EthR}$HEK-SEAP, transgenic for pWW489 and pWW491; see Fig. S4 a and b for clonal variation, Fig. S4c for adjustability, and Fig. S4d for reversibility of the gene circuit). $_{EthR}$HEK-SEAP cells were microencapsulated and implanted i.p. into mice. Animals treated with 2-phenylethyl-butyrate showed significantly reduced SEAP serum levels (without 2-phenylethyl-butyrate, 420 ± 43 milliunits/liter; with 625 μl/kg 2-phenylethyl-butyrate, 207 ± 29 milliunits/liter) suggesting that this compound was bioavailable and reached EthR-inactivating concentrations inside target cells. Mice with implanted control cells harboring only pWW491 showed background SEAP expression (84 ± 16 milliunits/liter).

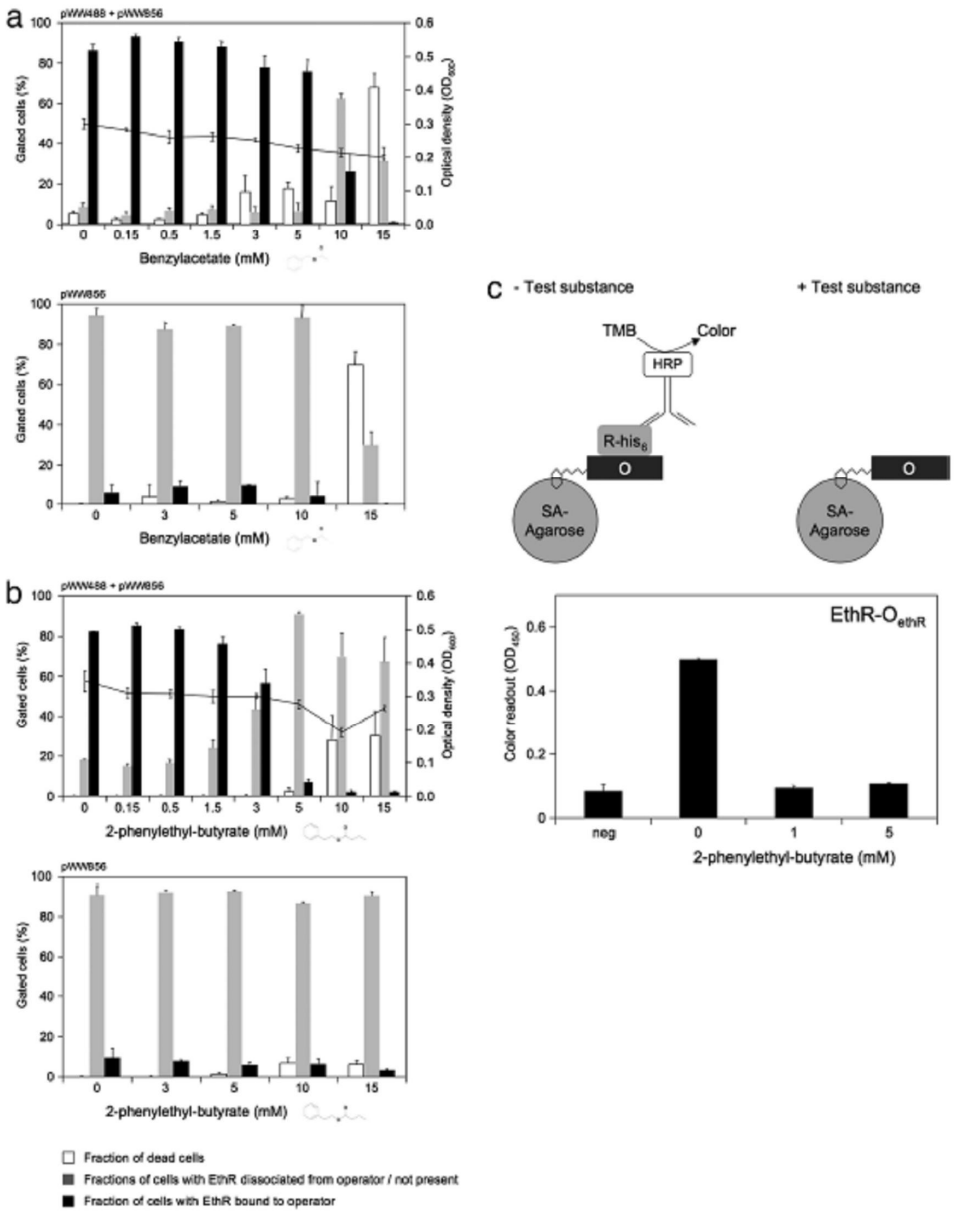

Fig. 2. Validation of the inducer in bacteria and in a cell-free system. (a) Effect of benzylacetate in *E. coli*. *E. coli* BL21(DE3), transformed with pWW488 and pWW856 (see Fig. S2a), was grown in the presence of IPTG (to induce EthR expression) at the indicated benzylacetate concentrations for 5.5 h before analyzing the cells by FACS (for FACS gates and parameters; see Fig. S2b). The optical density at 600 nm (OD_{600}) after the growth period is indicated as well. As a control, *E. coli* BL21(DE3) transformed with pWW856 alone were used in parallel. (b) Effect of 2-phenylethyl-butyrate in *E. coli*. Experimental setup as described in a. (c) Impact of 2-phenylethyl-butyrate on the interaction between EthR and O_{ethR} in vitro. Biotinylated operator O_{ethR} (O) immobilized on streptavidin-agarose beads (SA-Agarose) was incubated in the presence or absence of 2-phenylethyl-butyrate at the indicated concentrations in a cell lysate of *E. coli* BL21(DE3) transformed with pWW862 (P_{T7}-*ethR*-his_6-term) for production of hexahistidine-tagged EthR (R-his_6). After washing, his_6-tagged EthR was detected by a monoclonal anti-his_6 antibody coupled to horseradish peroxidase (HRP), resulting in the conversion of 3,3',5,5'-tetramethylbenzidine (TMB) to a colored formazane. As negative controls, nonrecombinant cell lysate was used (neg).

2-Phenylethyl-butyrate Increases the Sensitivity of *Mycobacterium bovis* and *M. tuberculosis* to Ethionamide. Growth of *M. tuberculosis* is significantly impaired in the presence of ethionamide because of EthA-mediated conversion of this prodrug into an antimycobacterial nicotinamide adenine dinucleotide derivative (3, 17). EthR-mediated repression of *ethA* transcription requires rather high clinical doses of ethionamide [up to 1 g/day (6, 18)], which is associated with severe side effects including neurotoxicity (19) and fatal hepatotoxicity (6), yet is often still insufficient to reach minimum inhibitory levels in the bloodstream (20). Therefore, 2-phenylethyl-butyrate-triggered dissociation of EthR from the *ethA* promoter resulting in derepression of *ethA*, which was confirmed by quantitative RT-PCR of *ethA* transcripts (2.93 ± 0.04- and 9.7 ± 1.7-fold increase in the presence of 0.5 and 2 mM 2-phenylethyl-butyrate, respectively), may increase the sensitivity of Mycobacterium to ethionamide-based therapy. Growth of *M. bovis* bacillus Calmette–Guérin and *M. tuberculosis* H37Rv in the presence of subinhibitory ethionamide concentrations (0.25 and 0.5 μg/ml), which are easily reached by therapeutic doses [c_{max} (250 mg oral) = 2 μg/ml; t1/2 = 2 h (18)] was dosedependently inhibited by 0.5 and 2 mM 2-phenylethyl-butyrate (Fig. 3). Because 2-phenylethyl-butyrate alone did not show any growth inhibitory effect, we suggest that it acted synergistically with ethionamide to kill the pathogen (Fig. 3). 4-Phenyl-2-butanone, which was previously suggested to neutralize EthR, was found to be cytotoxic (Fig. 1c and Fig. S1) and did not act synergistically with ethionamide to kill *M. bovis* at concentrations that were higher than the ones at which 2-phenylethyl-butyrate was effective (Fig. S5).

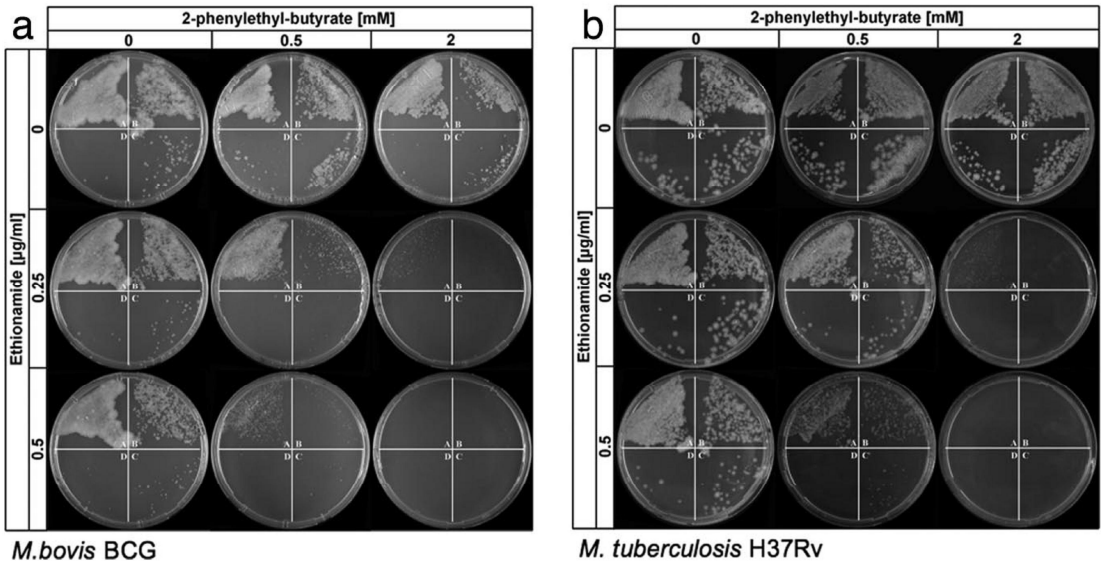

Fig. 3. Effect of 2-phenylethyl-butyrate and ethionamide on *M. bovis* bacillus Calmette–Guérin and *M. tuberculosis*. (a) Synergistic effect of 2-phenylethyl-butyrate and ethionamide (ETH) on the growth inhibition of *M. bovis* bacillus Calmette–Guérin. A–D correspond to serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) of an *M. bovis* bacillus Calmette–Guérin settling culture (OD_{600}: 0.6). (b) Synergistic effect of 2-phenylethyl-butyrate and ethionamide (ETH) on growth inhibition of *M. tuberculosis* H37Rv. A–D correspond to serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) of an *M. tuberculosis* H37Rv settling culture (OD_{600}: 0.4).

Discussion

Synthetic gene circuits have dramatically increased progress on gene-function relationships in the postgenomic era (8–11, 21– 25). They also continue to provide the key parts for synthetic biologists to decipher natural gene network dynamics (8–10) and to reprogram cellular function for production of important precursor drugs (23). We have engineered a synthetic gene network in human cells for screening of functional antimicrobial agents with precise target specificity, undetectable cytotoxicity, and the capacity to reach the cytosol to eliminate intracellular pathogens. The network setup is generic so that it can, in principle, be adapted to essential transcription regulators of other pathogens. With the integration of EthR (4), which controls the resistance of *M. tuberculosis* to ethionamide, into a synthetic mammalian gene network we have been able to identify the licensed food additive 2-phenylethyl-butyrate as a potent inhibitor of EthR in *M. tuberculosis*, as well as in vivo, which dramatically increases the sensitivity of this pathogen to the last-line-of-defense drug ethionamide and potentially to other *ethA*-dependent compounds (26). Therefore, 2-phenylethyl-butyrate could set up an efficient and safe line of defense against multidrug-resistant tuberculosis.

Materials and Methods

Vector Design. pWW489 ($P_{SV40\text{-}ethR}$-*vp16*-pA) was constructed by PCR-mediated amplification of *ethR* from genomic *M. bovis* DNA by using oligonucleotides OWW400 (5'-gcatccatatgaattccaccatgaccacctccgcggcca-3') and OWW401 (5'-cgatcgcgcgcggctgtac gcggagcggttctcgccgtaaatgc-3') followed by restriction and ligation (EcoRI/BssHII) into pWW35 (27). pWW491 ($O_{ethRPhsp70min}$- SEAP-pA) was obtained by direct cloning of a synthetic O*ethR* sequence (5'-gacgtcgatccacgctatcaacgtaatgtcgaggccgtcaacgagatgtc gacactatcgacacgtagcctgcagg-3') (AatII/SbfI) into pMF172 (27). pWW488 (PT7- *ethR-vp16*-his_6) was constructed by PCR-mediated amplification of *ethR-vp16* from pWW489 by using oligonucleotides OWW400 and OWW60 (5'- gctctagagcaagcttttaatggtgatg gtgatgatgcccaccgtactgtcaattccaag-3') followed by cloning (NdeI/HindIII) into pRSETmod (28). pWW856 (P_{ethR}-*gfp*-pA) was constructed in three steps: (i) *gfp* was PCR-amplified from pLEGFP-N1 (Clontech) by using oligonucleotides OWW848 (5'-ggcttgaattcaaa ggagatataccatggtgagcaagggcgag-3') and OWW849 (5'-ggctttctagacaaaaaacccctcaagacccgttta gaggccccaaggggttatgctagttacttgtacagctcgt ccatgccg-3') and cloned (EcoRI/XbaI) into pWW56 (27) (pWW854). (ii) A synthetic O_{ethR} sequence was directly cloned (HindIII/EcoRI) into pWW854 (pWW855). (iii) P_{ethR}*gfp* was excised (BamHI/StuI) from pWW855 and ligated (BamHI/ScaI) into pACYC177 (NEB) (pWW856). pWW862 (P_{T7}-*ethR*-his_6) was assembled by annealing oligonucleotides OWW479 (5'-cgcgca tcatcatcatcatcattaagcggccgca-3') and OWW480 (5'agcttgcggccgcttaatgatgatgatgatgatg-3') and cloning the double-stranded DNA BssHII/HindIII into pWW488. pWW871 (5'LTR-Ψ^+-*ethR-vp16*-P_{PGK}-neo^R-3'LTR) was designed by cloning *ethR-vp16* of pWW489 (EcoRI/ BamHI) into pMSCVneo (Clontech). pWW35 (P_{SV40}-E-*vp16*-pA), pWW37 (ETR$P_{hCMVmin}$-seap-pA) and pWW313 (P_{T7}-E-his_6-pA) have been described (28).

Cell Culture. Human embryonic kidney cells (HEK-293, American Type Culture Collection CRL-1573) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FCS (Pan Biotech GmbH, catalog no. 3302, lot P231902) and 1% of a penicillin/streptomycin solution (Sigma, catalog no. 4458). Cells were transfected by using standard calcium phosphate procedures (29) and retroviral particles were produced according to the manufacturer's protocol (Clontech). $_{EthR}$HEK, transgenic for constitutive EthR-VP16 expression, was constructed by transducing HEK-293 with pWW871- derived retroviral particles followed by selection in DMEM containing 200 μg/ml neomycin and single-cell cloning. Cotransfection of $_{EthR}$HEK with pWW491 and pPUR (Clontech), subsequent selection in 200 μg/ml neomycin, 1 μg/ml puromycin followed by single-cell cloning resulted

in $_{EthR}$HEK-SEAP. The cell line HEK-ET-SEAP, transgenic for constitutive SEAP expression, was described in ref. 27. SEAP was quantified in cell culture supernatants by using a p-nitrophenylphosphate- based assay (30) and in mouse serum by employing a commercial chemiluminescence test (Roche Applied Science, catalog no. 11779842001). The impact of chemicals on the viability of human cells was assessed by using the WST-1 cell proliferation assay according the to manufacturer's protocol (Roche Applied Science, catalog no. 05015944001).

Chemicals. Pentyl-acetate, methyl-phenylacetate, 2-phenylethyl-acetate, 4-phenyl-2-butanone (all Fluka) and 2-phenylethyl-butyrate (Sigma) were commercially obtained. Methyl-hexanoate was obtained by reacting hexanoic acid with thionylchloride in methanol. Butyl-propionate, 2-phenylethyl-propionate, 2-phenylethyl-isopentanoate, and 3-phenylpropyl-propionate were prepared by reacting the corresponding alcohol with the acid chloride in dichloromethane by using triethylamine as a base. Hexadecyloctanoate and benzylacetate were synthesized from the bromide and the acid with K_2CO_3 as the base in dimethylformamide. All esters were purified either by column chromatography (silica, ethylacetate/ hexane) or distillation. ClogP was determined by using Chemdraw Ultra 10.0 (CambridgeSoft). Erythromycin (Sigma, catalog no. E5389) was used as a 1,000x stock solution of 5 mg/ml in ethanol. Ethionamide was purchased from Sigma (catalog no. E6005) and prepared as 200x stock solution in DMSO.

FACS Analysis. *E. coli* BL21(DE3) (Invitrogen), transformed with pWW488 and pWW856 or pWW856alone, were grown overnight in Luria Bertani (LB) media containing 100 μg/ml ampicillin and 30 μg/ml kanamycin (for pWW856- transformed cells only). Then, 150 μl of *E. coli* suspension (OD_{600} 1.3) was added to 2 ml of fresh LB media containing antibiotics and 1 mM isopropyl β-D-thiogalactoside (IPTG) where indicated. After growth for 5.5 h at 37°C, 500 μl of the suspension was transferred to a new tube and centrifuged for 3 min at 800 x g. The pellet was washed twice with 1 ml of PBS and resuspended in 2 ml of PBS for FACS analysis (>10,000 cells per sample), which was performed on a Cytomics FC500 (Beckman Coulter) with 405 nm used for excitation and 510 nm for emission. FACS gates are shown in Fig. S2

ELISA. The EthR-his_6-specific ELISA was performed as previously described for the E-his_6-based ELISA (28) with the exception that the biotinylated operator sequence (O_{ethR}) was immobilized on streptavidin agarose beads (Novagen, catalog no. 69203) instead of a microtiter plate.

In Vivo Methods. $_{EthR}$HEK-SEAP was encapsulated in alginate-poly(L-lysine)- alginate capsules (200 cells per capsule) as described in ref. 27. Female OF1 (Oncins France souche 1, Charles River Laboratories) mice were injected i.p. with 700 μl of capsule suspension containing 2 x 10^6 cells. One and 25 h post-capsule implantation, the mice were injected with 2-phenylethyl-butyrate at the indicated concentration [the injection volume was adjusted to 100 μl by adding canola oil (Migros)]. Forty-eight hours post-capsule implantation, serum samples were analyzed for SEAP expression. Dissection of the animals revealed no inflammation at the injection site. Animal experiments were conducted by M.D.-E. at the Institut Universitaire de Technologie A (Lyon, France) in accordance with European Community legislation (86/609/ EEC) and approved by the French Republic (no. 69266310). For each experimental condition mean values including the standard deviation of at least eight mice are indicated.

Mycobacteria Cultivation and Susceptibility Testing. *M. tuberculosis* H37Rv (ATCC27294) and *M. bovis* bacillus Calmette–Guérin no. 1721, a streptomycin resistant derivative of bacillus Calmette–Guérin Pasteur, carrying a nonrestrictive *rpsL* mutation (K42R) (31) were grown in Middlebrook 7H9 supplemented with oleic acid, albumin, dextrose, catalase (Difco) and Tween 80 (0.05%) until midlog phase. Tenfold serial dilutions (20μl) were streaked on Middlebrook 7H10-OADC agar plates containing solvent (DMSO, 200-fold dilution), ethionamide (0.25–0.5 μg/ml) and 2-phenylethyl-butyrate (0.5 or 2 mM) where indicated. Plates were incubated at 37°C and growth was documented after 2 and 3 weeks. For quantitative analysis of *ethA* transcripts, *M. tuberculosis* H37Rv were treated at midlog phase with DMSO (40-fold dilution) or 2-phenylethyl-butyrate (0.5 or 2 mM) for 24 h at 37°C, harvested by centrifugation (4,400 x g, 10min, 4°C) and total RNA was extracted by using the RiboPure-Bacteria Kit (Ambion, catalog no. AM1925) according to the manufacturer's protocol. Total RNA was reverse transcribed (25°C, 10 min; 48°C, 30min; 95°C, 5min) by using TaqMan reverse transcription reagents (Applied Biosystems, catalog no. N8080234) and *ethA*-specific cDNA was quantified on an Applied Biosystems 7500 real-time PCR device by using the following *ethA*-specific primers: forward primer, 5'-cgatcacgacgatgttcttagc-3'; reverse primer, 5'-tcgggccgatcatccat-3'; probe labeled with 5'FAM and 3'TAMRA, 5'-FAM-cgtagtcgaggtcctcgggccagt-TAMRA-3'. All samples were standardized by using the *M. tuberculosis sigA* gene [Rv2703 (32)] and the following *sigA*-specific primers: forward primer, 5'-ccgatgacgacgaggagatc-3'; reverse primer, 5'-ggcctccgactcgtcttca-3'; probe labeled with 5'FAM and 3'TAMRA dyes, 5'-FAM-aaggacaaggcctccggtgatttcg-TAMRA-3'.

Acknowledgments. We thank Dominique Aubel for providing mycobacteria genomic DNA and generous advice, Martine Gilet for skillful assistance in animal experimentation, and David Greber for critical comments on the manuscript. This work was supported in part by the Swiss National Science Foundation (3100AO_120326; 3100AO_112549) and the European Union (LSHP-CT-2006-037217).

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Addendum

Personal contribution to chapter 5

In this article, a synthetic gene network in human cells was used for screening of a rationally designed chemical library with compounds which are sensing the interaction of EthR and its operator O_{ethR}. EthR is the repressor of *ethA*, whose gene-product EthA converts ETH, a second-line drug fighting the causative agent of Tuberculosis, into its active form with antimicrobial activity. The network identified the licensed food additive 2-phenylethyl-butyrate as a potent inhibitor of EthR in *M. tuberculosis* H37Rv and *M. bovis* BCG. The study showed that 2-phenylethyl-butyrate acts synergistically in combination with ETH in a way that growth of *M. tuberculosis* H37Rv and *M. bovis* BCG was inhibited in subinhibitory concentrations of ETH. The 2-phenylethyl-butyrate-triggered dissociation of EthR from the *ethA* promoter resulting in derepression of *ethA* was confirmed by quantitative RT-PCR of *ethA* transcripts.

My contribution as a co-author to this manuscript was as follows:

- Cultivation of *M. tuberculosis* H37Rv and *M. bovis* BCG, including design of the drug susceptibility tests with the two potential EthR-inhibitors 2-phenylethy-butyrate and 4-phenyl-2-butanone
- Isolation of total RNA of *M. tuberculosis* H37Rv
- Reverse transpcription total RNA to generate cDNA
- Writing of mycobateria-related part of the paper

Phenylethyl-butyrate enhances the effect of second-line drugs against clinical isolates of *M. tuberculosis*

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Antimicrob. Agents Chemother., in preparation.

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1. Abstract

The rise of Tuberculosis caused by multi-drug resistant strains of *M. tuberculosis* (MDR-TB) brings second-line drugs more and more into focus. One of these drugs is ethionamide (ETH). Being a prodrug itself, ETH has to be activated within the bacterium by the monooxygenase EthA. Expression of EthA is controlled by its natural repressor EthR and thus mycobacteria have an intrinsic resistance. Besides activating ETH, EthA also converts the prodrugs thiacetazone (TAC) and isoxyl (ISO) into their lethal form. These two antibiotics have been used in low-income countries as antitubercular drugs. Although all three drugs are activated by EthA, their molecular targets are divers. ETH inhibits the enoyl-acyl carrier protein reductase InhA, an enzyme involved in synthesis of mycolic acids, the essential components of the mycobacterial cell wall. TAC also affects mycolic acid biosynthesis but by inhibiting their cyclopropanation. ISO in turn affects mycolic acid synthesis by a yet not fully understood mechanism and additionally inhibits the stearoyl-CoA desaturase DesA3 which is involved in oleic- and tuberculostearic acid synthesis. We have shown previously that 2-phenylethyl-butyrate (2-PEB), a licensed food additive, efficiently inhibits EthR-binding and thereby enhances the expression of EthA. Therefore, a combination of 2-PEB and ETH has a synergistic effect on growth inhibition of *M. tuberculosis* laboratory strain H37Rv. Here we show that combinations of 2-PEB and either ETH, ISO or TAC enhance the growth inhibitory effect on *M. tuberculosis* H37Rv, on a set of drug susceptible and even on drug resistant clinical *M. tuberculosis* isolates.

2. Introduction

With more than 9.3 million new cases and more than 1.8 million deaths every year (24), tuberculosis (TB) still remains a global health problem. The recommended standard treatment to fight the causative agent *Mycobacterium tuberculosis* is a regimen of four out of five first line drugs isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (STR) for initial two months, followed by a four months treatment with INH and RIF. With the rise of drug-resistant strains of *M. tuberculosis*, the urge for new antibiotics or improved traditional antitubercular drugs is increasing. Multidrug-resistant strains (MDR) are resistant at least to the two first-line drugs INH and RIF (18) and require prolonged treatment with second line drugs, such as fluoroquinolones, aminoglycosides, ETH or *p*-aminosalicylic acid. However, extensively resistant strains (XDR) being additionally resistant to a fluorquinolone and one second-line injectable agent (amikacin, kanamycin or capreomycin) also occurred (10). Recently, even totally drug-resistant (TDR) strains have been reported (19), being resistant to all second-line antibiotics, including ethionamide (ETH). ETH is a pro-drug, which needs to be activated by the mycobacterial Baeyer-Villiger monooxygenase EthA to exert its antimicrobial activity by inhibiting the enoyl-ACP reductase InhA. ETH does not bind directly to InhA but forms a covalent adduct with NAD instead. This ETH-NAD adduct inhibits InhA efficiently (22). InhA, which is also a target of INH, is part of the fatty acid synthase type II system (FASII) which synthesizes mycolic acids, the essential components of the unique mycobacterial cell wall (21, 26). The expression of *ethA* is under control of its natural repressor EthR (3) which contributes to an increased intrinsic resistance and therefore ETH-based tuberculosis therapy is often unsuccessful, even when prescribed at high hepatotoxic doses (8). Acquired ETH-resistance is due to i) mutations in the *ethA*-gene and ii) mutations in the promoter region of the *mabA-inhA* operon (e.g. T-8C, T-8A, C-15T). Therefore, cross-resistance of the potent first-line drug INH and ETH are common, as mutations in *ethA* are a frequent mechanism of ETH-resistance in INH-resistant *katG*-mutants.

Besides ETH, thioamide drugs such as thiacetazone (TAC) and isoxyl (ISO) are also activated by EthA (6). TAC is an inexpensive, antitubercular, bacteriostatic drug that has widely been used in combination with INH in low-income countries. TAC has been replaced by EMB as it is often associated with dermatological side effects and Stevens-Johnson syndrome in AIDS patients with sometimes fatal skin reactions (9). In contrast to INH and ETH, which have a common target, the exact mechanism of action of TAC is not fully understood. It has been

shown that TAC alters the mycolic acid biosynthesis by inhibiting the cyclopropanating mycolic acid synthases (CMASs) (2). Interestingly, not all of the CMASs are inhibited by TAC. It has been found that MmaA4, a member of the CMAS family, is involved in the activation of the drug but is not inhibited. A new model has been proposed which claims that TAC has to be activated by EthA and MmaA4 to exert its fully lethal effect (1). Efforts have been made to synthesize chemical more effective TAC-analogues. Two of these analogues, SRI-224 and SRI-286, have been found to be more effective in growth inhibition of *M. avium* and *M. tuberculosis* than the parental drug (2, 4). ISO was used for the clinical treatment of TB in the 1960s. ISO monotherapy demonstrated modest efficacy in cases of pulmonary TB, but a combination of INH and ISO was more effective than treatment with either alone (15). Besides affecting the mycolic acid synthesis, ISO also targets the oleic acid synthesis (13). While the target in mycolic acid synthesis remains to be identified, ISO inhibits the stearoyl-CoA desaturase DesA3 and reduces therefore the amount of oleic- and tuberculostearic acid in mycobacteria (14). Although ISO has other targets than ETH and TAC it is activated by EthA as well (7, 11). Chemical analogues of ISO have been synthesized and tested against *M. tuberculosis* or *M. bovis* BCG. Modifications improved the activity of ISO against *M. tuberculosis*, MIC values nearly reached those of the first-line anti-tuberculosis drugs INH and RIF (5, 12).

The use of second line drugs like ETH, TAC and ISO or its derivatives often is accompanied by strong side effects. One of the reasons for those side effects is that the drugs have to be administered in high therapeutic doses to overcome the EthR-dependent EthA repression. However, it has been shown that chemical compounds like benzylacetate and 2-phenylethylbutyrate (2-PEB) inhibit EthR-binding and thus enhance the expression of EthA (23). In turn, increased expression of EthA enhances the antibacterial effect of ETH on *M. tuberculosis* H37Rv and *M. bovis* BCG. Also it has been shown, that substance BDM31343 exerts a synergistic effect with ETH in mice infected with *M. tuberculosis* laboratory strains H37Rv (25). Here we show that 2-PEB enhances the growth inhibitory effect of the three EthA-activated antibiotics ETH, ISO and TAC on *M. tuberculosis* H37Rv, on drug susceptible and drug resistant clinical isolates of *M. tuberculosis*.

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3. Materials and Methods

Strains

A total of 13 drug-susceptible, mono-resistant and multidrug-resistant clinical isolates of *M. tuberculosis* were studied. Strains 176914, 176861, 176747, 176587 and 176389 were sensitive to all first-line drugs. Strains 2694, 4269, 117 and 179987 were resistant to INH (0.1 µg/ml) due to a mutation in the promoter region of the *mabA-inhA* operon (C-15T). Strains 186038, 186137, 130 and 177836 were multidrug-resistant (INH, RIF) due to mutations in the *katG* gene and in the *rpoB* gene (S531L) (16, 17). As a control, the laboratory strain *M. tuberculosis* H37Rv (ATCC27294) was used.

Antimicrobial agents

ETH, TAC and 2-PEB were purchased from Sigma-Aldrich, ISO from Cayman-Chemical.

Molecular detection of resistance mutations

For the detection of mutational alterations associated with resistance to ETH, ISO and TAC, PCR-driven gene amplification and nucleic acid sequence determination of the *ethA*/*ethR* gene-region was applied. Primers 5'-GATGCAGAGGCGGTGTTC-3' and 5'-GTGTTCGGCGTCCACCCA-3' were used to amplify a 3.2 kbp fragment comprised of *ethA* (Rv3845c) and its upstream and downstream sequences. Amplified gene fragments were sequenced using the BigDye Terminator cycle-sequencing ready reaction kit (Applied Biosystems Inc.) and an ABI 3130 DNA genetic analyzer (Applied Biosystems Inc.).

Susceptibility testing using the MGIT 960 system with EpiCenter TB eXiST software

For drug susceptibility testing, the MGIT 960 system (Becton Dickinson) was used according to the manufacturers' manual. Briefly, 0.8 ml of MGIT 960 SIRE supplement (Becton Dickison) and 0.2 ml of the drug solution were added to the MGIT tubes. The tubes were inoculated with 0.5 ml of test strain suspension. As a control, a drug-free MGIT tube was inoculated with 0.5 ml of a 1:100 diluted (sterile H_2O) suspension of the test strain. Growth of the bacteria was monitored by the EpiCenter software (version 5.6.6), equipped with the TB eXiST module (Becton Dickinson) and was expressed as growth units (GU). A strain was considered to be resistant (R) to a drug when the test tube reached ≥100 GU earlier than the

drug-free control tube reached a GU-value of 400. Sensitivity (S) of a strain was defined when the control tube reached 400 GU and the test tube remained ≤100 GU for more than 7 days after the control tube has reached 400 GU. A strain was considered to be intermediate (I) when the test tube reached ≥100 GU within 7 days after the control tube reached 400 GU.

4. Results

Effect of 2-PEB in combination with ETH on growth of drug-susceptible *M. tuberculosis*. In an initial phase, all strains have been challenged with 5 different concentrations of ETH, ranging from 12.5 μg/ml to 0.16 μg/ml in combination with 0.75 mM 2-PEB and without the addition of 2-PEB as a control. Table 1 indicates that all (6/6) drug-susceptible clinical isolates and *M. tuberculosis* H37Rv show a synergistic effect of ETH and 2-PEB. These strains change their resistance profile by shifting from resistance (R) to intermediate (I) or I to sensitive (S). The lowest concentration of ETH on which 2-PEB showed a synergistic effect was 0.625 μg/ml, below this concentration no additional benefit could be detected. Also, 2-PEB alone does not induce a growth inhibition effect as the resistance profile shows no shifting with the compound alone (data not shown).

In a second phase, 8 strains being resistant to at least one of the first-line drugs have been tested. The strains have been challenged with 5 concentrations of ETH, ranging from 12.5 μg/ml to 1.25 μg/ml, with and without adding 0.75 mM 2-PEB. Table 1 shows that 2/8 of the drug-resistant strains (2694 and 117) showed a synergistic effect of ETH and 2-PEB. In both cases, the shift from R to either I (2694) or S (117) can be seen at an ETH-concentration of 5 μg/ml. At lower concentrations no synergistic effect of 2-PEB can be detected.

Effect of 2-PEB in combination with ISO and TAC on growth of *M. tuberculosis*. As ISO and TAC share the same activator as ETH (6), possible synergistic effects of 2-PEB have been examined in combination with these two antibiotics. A subset of the above tested strains (176914, 176747, 2694, 4269 and 117) has been chosen for challenging in the MGIT-system with the following concentrations: ISO: 2.7, 0.9, 0.3 and 0.1 μg/ml and TAC: 0.66, 0.22, 0.08 and 0.03 μg/ml. All concentrations have been tested with and without the addition of 0.75 mM 2-PEB. Four out of 5 strains (176914, 2694, 4269 and 117) showed an increased susceptibility to ISO when adding 2-PEB (Table 1). Remarkably, three of these strains are resistant to the major first-line drug INH. Strain 2694 showed a synergism in combination with 2-PEB at a concentration of 0.3 μg/ml ISO, while strains 4269 and 117 showed a synergism at 0.9 μg/ml instead. A synergistic effect of 2-PEB with TAC was observed in 4/5 strains (176914, 176747, 2694 and 117) while strain 4269 is resistant to TAC, even at the highest concentration of 0.66 μg/ml, also tested in combination with 2-PEB (Table 1).

Molecular detection of mutations in the *ethR/ethA* region. In order to elucidate the molecular mechanism of resistance to ETH particularly in those strains without an *inhA*-promoter mutation, we amplified and sequenced the *ethR*/*ethA* gene-region of all above tested strains. All INH-sensitive strains showed a wild-type *ethR*/*ethA* sequence (Table 1). Of the

INH low-level resistant strains, i.e. those with an *inhA*-promoter mutation C-15T only one strain (4269) exhibited an altered *ethR*/*ethA* sequence, due to two nucleotide exchanges. One of the mutations is silent (TCG1326TCT, S442S), the second leads to an Asn to Lys conversion at position 345. The strains which are high-level resistant to INH because of the mutation S315T1 in the according activator KatG also all carry mutations in *ethA*. Only one mutation in strain 130 turned out to be silent (CCA771CCG, P257P), while the other mutations affect the EthA protein. Strains 186137 and 186036 do not express fully mature EthA, either because of a frameshift mutation at position 7 or a conversion of Trp to Stop in case of 186038. Strain 177836 has a point mutation which leads to conversion of a Ser into an Arg at position 256.

5. Discussion

The global rise of MDR and XDR strains indicates the increasing need for new treatment regimens of patients with TB. The three antitubercular drugs ETH, ISO and TAC are considered as a last line of defense to fight TB. These antibiotics are pro-drugs and have to be activated within the bacterial cell. ETH, ISO and TAC share the same activator EthA (6). The expression of EthA is under control of its natural repressor EthR (3). Previous studies showed that several compounds prevent EthR from binding to the *ethA* promoter, e.g. 2-PEB (23) or BDM31381 (25). Additives like 2-PEB therefore have the potential to reduce the therapeutic dose of those antibiotics and thereby would be able to decrease the severe side effects.

Here we demonstrate that the sensitivity of thioamide drugs like ethionamide, isoxyl and thiacetazone is increased by 2-PEB, also when applying to drug resistant strains of *M. tuberculosis*. Upon co-administration of 2-PEB together with ETH, all (6/6) drug-sensitive strains and 2/4 INH-resistant strains with a C-15T mutation showed a synergism. All MDR-strains (130, 186137, 186038 and 177836) with mutations in *katG* did not show a synergism when administering ETH and 2-PEB. As a matter of fact, all MDR-strains carry mutations in *ethA* additionally to the *katG* mutations. The most severe mutations were found in strain 186137, which has a frameshift mutation at position 7, and in strain 186038, which encodes a stop instead of a tryptophan at position 256. Hence, mutations in *ethA* are a frequent mechanism of ETH resistance in high-level INH-resistant strains with *katG* mutants. Upon co-administration of 2-PEB and ISO, one of two drug-sensitive strains and three out of three drug-resistant strains had an enhanced sensitivity towards ISO. The growth inhibitory effect of TAC was enhanced in two out of two drug-sensitive strains when applying 2-PEB, while the same effect was seen in two out of three drug-resistant strains. However, not all strains tested showed the same resistance profile and the same behavior concerning the synergism with 2-PEB. Even though we analyzed the gene sequence of the *ethR*/*ethA*-region, not every resistance phenotype could be explained. Remarkably, no mutation has been found in the region of *ethR*, which could lead to a higher binding capacity of EthR and therefore lower the transcription of the *ethA*-gene. We found that strain 130, which neither carries a mutation in the *inhA* promoter region nor in the *ethA*-gene but anyhow is resistant to ETH. This may be explained by recent findings, that not only EthA is responsible for the activation of ETH. Vilchèze et al. (20) showed that the activation of ETH is depending as well on a functional *mshA*-gene, which is involved in the production of mycothiol and therefore plays a role in the pro-drug activation by *ethA*.

One strain (176747) showed a synergistic effect of either ETH or TAC together with 2-PEB but not when administering ISO. Probably, the affinity of EthA for ISO is much higher than its affinity for ETH. The basal level of EthA produced in the bacterium in some cases might be sufficient to activate ISO very efficiently but not sufficient for ETH. This might support earlier observations of Dover et al. (7). The study shows that the overproduction of EthA in an *ethR* knock-out strain of *M. bovis* BCG does not result in significantly higher yields of activated drug in comparison to the parental strain. The low -hypersensitivity effect of ISO may be linked to the instrinsic structure of ISO, which is very efficiently metabolized by endogenous EthA. Boosting TAC with 2-PEB worked well with the exception of strain 4269 which was completely resistant to all applied concentrations. As this strain is resistant to ETH even at the highest concentration and it carries a mutation in the *ethA*-gene (N245K) the resistance phenotype can be explained. Nevertheless, isolation of spontaneous mutants in *Mycobacterium bovis* BCG that are highly resistant to TAC but carry a functional EthA has been reported recently (1). These TAC-resistant strains had mutations in *mmaA4*, a gene encoding for a methyltransferase, which is required for synthesis of keto- and methoxy-mycolic acids. Therefore these mutants lacked keto-mycolic acids, which are long-chain fatty acids associated with the cell wall. The drug-sensitive phenotype could be restored by complementation with a functional *mmaA4* gene. As these mutants had an increased susceptibility to TAC upon overexpression of MmaA4, it has been hypothesized that a functional MmaA4 is necessary for activation of the pro-drug, rather than being a target for TAC. An altered MmaA4 may explain the resistance of strain 4269 to TAC while being still sensitive to ISO.

The notion that chemical analogues of both drugs, ISO and TAC, are available and showed to be more efficient than the parental drugs is very intriguing. It might be interesting to test whether they are boosted by 2-PEB as well and therefore may be developed as valuable alternatives for existing second-line drugs but with lower side-effects. Willand et al. (25) showed lately that substance BDM31381 is able to inhibit EthR and hence boost the antibacterial activity ETH on a laboratory strain of *M. tuberculosis in vitro* and in mouse infection experiments. They also showed that the growth inhibition effect of TAC can be enhanced by BDM31381 but the enhancement of ISO failed. A possibility to improve the efficacy of the already licensed food additive 2-PEB (Joint Food and Agriculture Organization (FAO)/WHO Expert Committee on Food Additives, JEFCA no. 991), is the application of further rational drug design by synthetic mammalian gene circuits (23).

In conclusion, our data show that it is possible to enhance the growth inhibitory effect of the three thioamide drugs ethionamide, isoxyl and thiacetazone by adding 2-PEB. Most interestingly, this synergistic effect has been demonstrated against *M. tuberculosis* H37Rv, several drug-sensitive clinical isolates and, most important, also to drug-resistant strains of *M. tuberculosis*. Our findings might contribute to a re-consideration of ETH, ISO and TAC as low-cost second-line drugs, which are getting more important because of emerging MDR- and XDR strains of *M. tuberculosis*. Additionally, the ability of 2-PEB to lower the applied concentrations of the antibiotics might reduce the side effects of these drugs and thus improve patients well-being. However, the *ethA* locus should be characterized, particularly in high level INH resistant strains.

6. Acknowledgments

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Strain	ETH				ISO				TAC				Genetic resistance		
	Conc. [µg/ml]	w/o PEB	PEB	Synergism	Conc. [µg/ml]	w/o PEB	PEB	Synergism	Conc. [µg/ml]	w/o PEB	PEB	Synergism	*katG*	*inhA*	*ethA*
H37Rv	12.5	S	S	No									wt	wt	wt
	5	S	S	No											
	2.5	S	S	No											
	1.25	I	S	Yes											
	0.625	R	I	Yes											
	0.31	R	R	No											
	0.16	R	R	No											
176914	12.5	I	S	Yes									wt	wt	wt
	5	I	S	Yes											
	2.5	R	I	Yes											
	1.25	R	I	Yes	2.7	S	S	No	0.66	S	S	No			
	0.625	R	I	Yes	0.9	S	S	No	0.22	S	S	No			
	0.31	R	R	No	0.3	I	I	No	0.08	I	S	Yes			
	0.16	R	R	No	0.1	R	I	Yes	0.03	R	I	Yes			
176861	12.5	I	I	No									wt	wt	wt
	5	I	I	No											
	2.5	I	I	No	n.d.				n.d.						
	1.25	R	I	Yes	n.d.				n.d.						
	0.625	R	I	Yes	n.d.				n.d.						
	0.31	R	R	No	n.d.				n.d.						
	0.16	R	R	No	n.d.				n.d.						
176747	12.5	S	S	No									wt	wt	wt
	5	I	S	Yes											
	2.5	R	I	Yes											
	1.25	R	I	Yes	2.7	S	S	No	0.66	R	I	Yes			
	0.625	R	R	No	0.9	I	I	No	0.22	R	R	No			
	0.31	R	R	No	0.3	R	R	No	0.08	R	R	No			
	0.16	R	R	No	0.1	R	R	No	0.03	R	R	No			
176587	12.5	I	S	Yes									wt	wt	wt
	5	I	I	No											
	2.5	I	I	No	n.d.				n.d.						
	1.25	I	I	No	n.d.				n.d.						
	0.625	R	I	Yes	n.d.				n.d.						
	0.31	R	R	No	n.d.				n.d.						
	0.16	R	R	No	n.d.				n.d.						
176389	12.5	I	S	Yes									wt	wt	wt
	5	I	I	No											
	2.5	I	I	No	n.d.				n.d.						
	1.25	I	I	No	n.d.				n.d.						
	0.625	R	I	Yes	n.d.				n.d.						
	0.31	R	R	No	n.d.				n.d.						
	0.16	R	R	No	n.d.				n.d.						

2694	12.5	S	S	No	2.7	S	S	No	0.66	I	S	Yes	wt	C-15T	wt
	5	R	S	Yes	0.9	S	S	No	0.22	I	S	Yes			
	2.5	R	R	No	0.3	R	I	Yes	0.08	R	R	No			
	1.25	R	R	No	0.1	R	R	No	0.03	R	R	No			
4269	12.5	R	R	No	2.7	I	I	No	0.66	R	R	No	wt	C-15T	N345K
	5	R	R	No	0.9	R	I	Yes	0.22	R	R	No			S442S
	2.5	R	R	No	0.3	R	R	No	0.08	R	R	No			
	1.25	R	R	No	0.1	R	R	No	0.03	R	R	No			
117	12.5	S	S	No	2.7	S	S	No	0.66	R	S	Yes	wt	C-15T	wt
	5	R	I	Yes	0.9	I	S	Yes	0.22	R	S	Yes			
	2.5	R	R	No	0.3	R	R	No	0.08	R	R	No			
	1.25	R	R	No	0.1	R	R	No	0.03	R	R	No			
179987	12.5	S	I	No	n.d.				n.d.				wt	C-15T	wt
	5	R	R	No	n.d.				n.d.						
	2.5	R	R	No	n.d.				n.d.						
	1.25	R	R	No	n.d.				n.d.						
130	12.5	R	R	No	n.d.				n.d.				S315T1	wt	P257P
	5	R	R	No	n.d.				n.d.						
	2.5	R	R	No	n.d.				n.d.						
	1.25	R	R	No	n.d.				n.d.						
186137	12.5	R	R	No	n.d.				n.d.				S315T1	wt	Fs pos. 7
	5	R	R	No	n.d.				n.d.						
	2.5	R	R	No	n.d.				n.d.						
	1.25	R	R	No	n.d.				n.d.						
186038	12.5	S	R	No	n.d.				n.d.				S315T1	wt	W156Stop
	5	R	R	No	n.d.				n.d.						
	2.5	R	R	No	n.d.				n.d.						
	1.25	R	R	No	n.d.				n.d.						
177836	12.5	R	R	No	n.d.				n.d.				S315T1	wt	S266R
	5	R	R	No	n.d.				n.d.						
	2.5	R	R	No	n.d.				n.d.						
	1.25	R	R	No	n.d.				n.d.						

TABLE 1. Synergistic effects of ETH, ISO and TAC with 2-PEB on growth of clinical isolates of *M. tuberculosis*

Addendum

Personal contribution to chapter 6

In this article it was shown that the licensed food additive 2-phenylethyl-butyrate (2-PEB) was able to enhance the efficacy of important second-line drugs such as ethionamide (ETH), isoxyl (ISO) and thiacetazone (TAC). These pro-drugs are converted by a common activator, EthA. The gene which encodes this Baeyer-Villiger monooxygenase is naturally repressed by EthR. By applying 2-PEB, which is able to release EthR from the *ethA*-promoter, the antibacterial effect of ETH, ISO and TAC was enhanced. This synergistic effect was achieved when challenging *M. tuberculosis* strain H37Rv, clinical isolates of *M. tuberculosis* fully susceptible to all common antibiotics and drug-resistant strains with a resistance towards ETH.

My contribution as first author to this manuscript was as follows:

- Design of the study
- MIC-determination
- Sequencing of the *ethR*/*ethA* gene
- Determination of synergistic effects with the MGIT-system
- Writing of the manuscript

Drug Screening NAD(P) Biosynthesis Pathway

NAD-synthetase Inhibitors 1st generation

NAD^+ is an essential metabolite, and its biosynthetic pathways are of major interest for the development of new drugs. In contrast to humans, where an NAD^+ synthetase-independent recycling pathway exists, *M. tuberculosis* NAD^+ biosynthesis is absolutely dependent on the activity of NAD^+ synthetase. Therefore 11 candidate compounds with proven inhibition of the NAD(P)H biosynthesis pathway in a target based (biochemical) assay (Hegymegi-Barakonyi *et al.*, 2008, M. Rizzi, personal communication) have been evaluated in a whole cell assay with respect to growth inhibition of *M. tuberculosis* laboratory strain H37Rv and a multidrug-resistant clinical isolate. The clinical isolate is resistant to the following antibiotics: Ethambutol (5 μg/ml), ethionamide (2.5 μg/ml), isoniazid (0.1, 1.0, 3.0, 10.0 μg/ml), rifampicin (1.0, 10.0, 50.0 μg/ml) and streptomycin (1.0, 20.0 μg/ml) due to mutations in the following genes: *rpoB* (S531L), *katG* (S315T1) and *inhA* (C15T). Possible growth inhibitory effects have been tested using the Becton Dickinson BBL™ MGIT™ 960 system (MGIT) in a BSL3 laboratory. The following 11 candidate compounds have been tested in concentrations of 5, 50 and 500 μM: 6026, 6569, 11085, 12507, 12539, 13632, 15108, 16047, 16315, 16581, and 17731.

At a concentration of 5 μM, no compound showed a growth inhibitory effect, neither in the drug-sensitive laboratory strain H37Rv, nor in the multidrug-resistant patient isolate (Table 1). Compound 15108 is the most effective inhibitor in terms of growth inhibition effect. It inhibited growth of both strains by >99% at a concentration of 50 μM, while none of the other 10 compounds did show any effect at this concentration. The following 6 compounds showed a growth inhibitory effect at a concentration of 500 μM in all three experiments: 12507, 12539, 13632, 15108, 16315 and 17731. Compound 16581 showed a growth inhibition effect of >99% in experiments with the laboratory strain H37Rv and the clinical isolate. Several compounds inhibited growth completely while 4 compounds did not show any effect on growth of either H37Rv or the clinical isolate (Table 1).

		Time [d] until reaching growth units threshold					
Compound	Concentration [µM]	1st experiment (H37Rv)		2nd experiment (H37Rv)		3rd experiment (MDR)	
		>100	>400	>100	>400	>100	>400
Growth control		3.25	3.75	3.3	3.8	3.3	3.9
Solvent		3.25	3.75	3.25	3.8	3.2	3.75
Growth control (1:100)#		5.5	6.5	6.75	7.8	7.75	8.5
6026	5	2.8	3.5	3.75	4.25	3.2	3.8
	50	3.5	4.1	3.75	4.25	3.5	4.1
	500	3.6	4.25	3.9	4.6	3.6	4.25
6569	5	3.5	3.7	3.25	3.8	2.2	2.3
	50	3	3.6	3.5	4.2	3.25	3.8
	500	3.3	4.2	3.8	4.6	3.7	4.3
11085	5	2.8	3.6	3.25	3.9	3.4	3.9
	50	3.1	3.8	3.25	3.9	3.5	4.1
	500	3.8	4.5	4.4	5.2	3.8	4.7
12507	5	2.9	3.7	3.7	4.3	3.5	4.1
	50	3.7	4.25	4.1	4.75	4.1	4.75
	500	>21#	>21#	>20#	>20#	>17#	>17#
12539	5	3.4	3.8	3.5	4.2	3.7	4.25
	50	3.6	4.3	3.6	4.25	4.5	5.2
	500	>21#	>21#	>20#	>20#	>17#	>17#
13632	5	3	3.6	3.6	4.25	3.3	3.9
	50	4	4.75	4.5	5.25	4.6	5.4
	500	7#	8.1#	8.25#	9.3#	15.25#	16.25#
15108‡	5	3.2	3.7	3.75	4.3	2.4	2.5
	50	6.25#	7.4#	5.5	6.75	8.6#	10.6#
	500	>21#	>21#	>20#	>20#	>17#	>17#
16047	5	3.3	3.8	3.75	4.25	3.25	3.8
	50	3.5	4	3.75	4.25	3.4	4
	500	4.2	4.8	4.3	5	4.5	5.2
16315‡	5	3.4	3.9	3.6	4.2	3.25	3.8
	50	4.2	4.8	4.5	5.3	5	5.75
	500	7.25#	8.4#	8.8#	9.8#	9.25#	10.6#
16581‡	5	3	3.5	3.25	3.9	3.5	4
	50	3.25	3.8	4	4.7	3.9	4.7
	500	>21#	>21#	5.25	6.2	>17#	>17#
17731	5	3.25	3.8	3.5	4.2	3.2	3.75
	50	3.1	3.6	3.4	4.1	3.7	4.25
	500	7.4#	9.25#	6.75#	8.75#	>17#	>17#
First/second line drug§	low	14.25	15.75	12.25	13.4	>17	>17
	high	14.6	17	>20	>20	>17	>17

Table 1: Drug susceptibility testing of potential NAD-synthetase inhibitors. # indicates concentrations of compounds with >99% growth inhibition (in comparison to 1:100 growth control). § First line drug for H37Rv; INH (low 0.1 µg/ml; high: 1.0 µg/ml). For MDR: AK (low: 1.0 µg/ml; high: 4.0 µg/ml).

NAD-synthetase Inhibitors 2nd generation

As a follow-up of the 1st generation compounds, 2nd generation compounds have been synthesized and tested in a target based (biochemical) assay (M. Rizzi, personal communication). Six of these compounds (12601, 12974, 13256, 13268 and 16103) have been tested with respect to growth inhibitory effect using the BBL™ MGIT™ 960 system. As these compounds did not show any growth inhibitory effect at a concentration of 100 µM (data not shown), they have been tested at a concentration of 500 µM (Table 2)

Compound concentration: 500 µM	Compound	Time [d] until reaching growth units threshold		Conclusion
	Number	>100	>400	
	Growth control	3	3.5	
	Solvent	2.9	3.3	
Inhibition 0-99%	12601	5.5	8	no effect
99% Inhibition Control	Growth control (1:100)	6.3	7	
Inhibition >99%	12974	>21	>21	effective
	13256	>21	>21	
	13268	8.7	11.6	
	16103	>21	>21	
First line drug	INH (0.1 µg/ml)	12.4	13.3	
	INH (1.0 µg/ml)	13.4	15	

Table 2: Results drug screening NAD(P) biosynthesis pathway, second generation compounds (500 µM)

At a concentration of 500 µM, 3 out of 5 compounds (12974, 13256, 16103) showed a complete growth inhibition, 1 compound (13268), reduced growth by >99%. One compound (12601) did not reduce growth at all.

NAD-synthetase MIC determination of compound 15108

The most effective NAD-synthetase inhibitor 15108 of the first generation compounds has been tested in 6 different concentrations, reaching from 5 up to 80 µM in the BBL™ MGIT™

960 system. Compound 15108 reduced growth by >99% starting at a concentration between 40 µM and 60 µM (Table 3).

Compound 15108		Time [d] until reaching growth units threshold		Conclusion
	Compound concentration	>100	>400	
	Growth control	3	3.5	
	Solvent	2.9	3.3	
Inhibition 0-99%	5 µM	3.1	3.5	no effect
	10 µM	3.25	3.8	
	20 µM	3.7	4.3	
	40 µM	5.2	6.4	
99% Inhibition Control	Growth control (1:100)	6.3	7	
Inhibition >99%	60 µM	8.3	9.8	effective
	80 µM	21.2	23.9	
First line drug	INH (0.1 µg/ml)	12.4	13.3	
	INH (1.0 µg/ml)	13.4	15	

Table 3: MIC-determination of NAD-synthetase inhibitor 15108

Synergistic effects of compound 15108 with different classes of antibiotics

According to a recent publication (Kohanski *et al.*, 2007), bactericidal but not bacteriostatic antibiotics induce hydroxyl radical formation, which causes serious damage in Gram-positive as well as in Gram-negative bacteria, leading to cell death. The augmented hydroxyl radical formation is based on a rapid depletion of NADH upon administration of bactericidal drugs. As the effect of 15108 as an NAD-synthetase inhibitor leads to a lower level of NADH, the synergistic effect of 15108 together with different antibiotics has been tested. The following antibiotics as representatives of their classes have been tested in the BBL™ MGIT™ 960 system: Ofloxacin (quinolone, bactericidal, 0.25 µg/ml), amikacin (aminoglycoside, bactericidal, 0.25 µg/ml), rifampicin (bacteriostatic 0.25 µg/ml) and isoniazid (bacteriostatic 0.025 µg/ml). All antibiotics have been applied in sub-inhibitory concentrations. The NAD-synthetase inhibitor 15108 was tested in two different concentrations, 3.0 and 15.0 µM, both below the MIC of 60 µM evaluated in section 4.4.3. As controls, 15108 has been tested at concentrations of 3.0 and 15.0 µM without adding an antibiotic and all antibiotics have been

tested without the addition of 15108 at the above mentioned concentrations. Table 4.4 shows the results of the synergism-testing.

Synergistic effect of NAD-synthetase inhibitor 15108 and several different antibiotics on growth of *M. tuberculosis* H37Rv

15108 [µM]	without antibiotics	Ofloxacin (0.25 µg/ml)	Amikacin (0.25 µg/ml)	Rifampicin (0.25 µg/ml)	Isoniazid (0.025 µg/ml)
0.0	R	R	R	R	R
3.0	R	R	R	R	R
15.0	R	R	R	R	R
60.0	S	n.d.	n.d.	n.d.	n.d.

Table 4: Synergistic effects of NAD-synthetase inhibitor 15108 and several antibiotics on growth of *M. tuberculosis* H37Rv

The first-line antibiotics RIF and INH did not show a synergistic effect when applied in combination with 15108. Likewise, the second-line drugs OFL and AMK did not exert a synergistic effect on growth of *M. tuberculosis* H37Rv in combination with 15108 as well, indicated with R in table 4.

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Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A. & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**, 797-810.

List of Publications

2010 **Grau, T.*,** Tschumi, A.*, Burri, R., Brülle, J. K. & Sander, P. (2010). Cell wall transport of mycobacterial lipoproteins depends on an N-terminal translocation signal. In preparation.

** Thomas Grau and Andreas Tschumi contributed equally to this work*

Tschumi, A.*, **Grau, T.***, Antelmann, H., Selchow, P. & Sander, P. (2010). Characterization of the gating enzyme of the mycobacterial lipoprotein biosynthesis pathway - prolipoprotein diacylglyceryl transferase Lgt. In preparation.

** Thomas Grau and Andreas Tschumi contributed equally to this work*

Grau, T., Gitzinger, M., Ritter, C., Burri, R., Selchow, P., Böttger, E. C., Fussenegger, M., Weber, W. & Sander P. (2010). Phenyethyl-butyrate enhances the effect of second-line drugs against clinical isolates of *M. tuberculosis*. *Antimicrob. Agents Chemother.*, in preparation.

Brülle, J. K.*, **Grau, T.***, Tschumi, A., Auchli, Y., Burri, R., Polsfuss, S., Keller, P. M., Hunziker, P. & Sander, P. (2010). Cloning, expression and characterization of *Mycobacterium tuberculosis* lipoprotein LprF. *Biochem. Biophys. Res. Commun.* 391, 679-684.

** Juliane K. Brülle and Thomas Grau contributed equally to this work*

2009 Tschumi, A., Nai, C., Auchli, Y., Hunziker, P., Gehrig, P., Keller, P., **Grau, T.** & Sander P. (2009). Identification of Apolipoprotein *N*-Acyltransferase (Lnt) in Mycobacteria. *J. Biol. Chem.* 284, 27146-27156.

2008 Weber, W., Schoenmakers, R., Keller, B., Gitzinger, M., **Grau, T.**, Daoud-El Baba, M., Sander, P. & Fussenegger, M. (2008). A synthetic mammalian gene circuit reveals antituberculosis compounds. *Proc. Natl. Acad. Sci. USA* 105, 9994-9998.

2007 Rezwan, M., **Grau, T.**, Tschumi, A. & Sander P. (2007). Lipoprotein synthesis in mycobacteria (Mini-Review). *Microbiology* 153, 652-658.

2002 Koller, M. F.*, **Grau, T.*** & Christen, P. (2002). Induction of antibodies against murine full-length prion protein in wild-type mice. *J. Neuroimmunol.* 132, 113-116.

** Michael F. Koller and Thomas Grau contributed equally to this work.*

Awards

2009 The publication "A synthetic mammalian gene circuit reveals antituberculosis compounds", *Proc. Natl. Acad. Sci. USA 105, 9994-9998*, has been awarded with the "SwissTB Award" by the Swiss Foundation for Tuberculosis Research.

Oral and Poster Presentations

Short Lectures

2010 Annual Meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM) and 62nd Annual Meeting of the Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM), Hannover, Germany

Dissection of the lipoprotein synthesis pathway in mycobacteria

T. Grau, A. Tschumi, S. Polsfuss, J. K. Brülle, P. M. Keller, P. Selchow, T. Rosenberger, A. Petrera, B. Amstutz & P. Sander*

2009 61st Annual Meeting of the Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM), Göttingen, Germany

Molecular characterization of Mycobacterium tuberculosis lipoprotein LppX

A. Tschumi, C. Nai, Y. Auchli, P. Hunziker, P. Gehrig, P. Keller, **T. Grau** & P. Sander*

2008 67th Annual Assembly of the Swiss Society for Microbiology (SSM), Interlaken, Switzerland

Drug Discovery: Development of lipoprotein signal peptidase activity assay

M. Dolder, P. Keller*, **T. Grau**, A. Tschumi & P. Sander

* Presenting author

Poster Presentations

2010 69th Annual Assembly of the Swiss Society for Microbiology (SSM), Zurich, Switzerland

Phenylethyl-butyrate enhances the effect of second-line drugs against clinical isolates of M. tuberculosis

T. Grau*, M. Gitzinger, C. Ritter, R. Burri, P. Selchow, M. Fussenegger, E. C. Böttger & P. Sander

The lipid anchor of mycobacterial lipoproteins

J. Brülle*, A. Tschumi, **T. Grau**, Y. Auchli, P. Hunziker & P. Sander

2009 68th Annual Assembly of the Swiss Society for Microbiology (SSM), Lausanne, Switzerland

A synthetic mammalian gene circuit reveals antituberculosis compounds

W. Weber, R. Schoenmakers, B. Keller, M. Gitzinger, **T. Grau***, M. Daoud El-Baba, P. Sander & M. Fussenegger

Identification of apolipoprotein N-acyltransferase activity in mycobacteria

A. Tschumi*, C. Nai, Y. Auchli, P. Hunziker, P. Gehrig, P. Keller, **T. Grau** & P. Sander

2008 67th Annual Assembly of the Swiss Society for Microbiology (SSM), Interlaken, Switzerland

Subcellular localization of mycobacterial lipoproteins

T. Grau*, A. Tschumi, P. Keller, T. Rosenberger, E. C. Böttger & P. Sander

Characterization of Mycobacterium tuberculosis LpqE

P. Keller*, **T. Grau** & P. Sander

Ppm1 is required for virulence Mycobacterium tuberculosis

A. Tschumi*, P. Selchow, **T. Grau**, K. Walther, S. Ehlers & P. Sander

* Presenting author

2007 Keystone Symposia on Molecular and Cellular Biology. Tuberculosis: From Lab Research to Field Trials, Vancouver, Canada

Lipoprotein transport to the cell wall of mycobacteria

T. Grau*, A. Tschumi, M. Rezwan, M. Dolder, S. Gempeler, E. C. Böttger & P. Sander

Lipoprotein synthesis in mycobacteria

A. Tschumi*, M. Rezwan, **T. Grau**, S. Gempeler, P. Keller & P. Sander

66th Annual Assembly of the Swiss Society for Microbiology (SSM), Interlaken, Switzerland

Lipoprotein transport to the cell wall of mycobacteria

T. Grau*, A. Tschumi, M. Rezwan, M. Dolder, S. Gempeler, E. C. Böttger & P. Sander

Lipoprotein synthesis in mycobacteria

A. Tschumi*, M. Rezwan, **T. Grau**, S. Gempeler, P. Keller & P. Sander

2005 64th Annual Assembly of the Swiss Society for Microbiology (SSM), Geneva, Switzerland

The lipoprotein modifying enzyme Lnt is found in organisms with a lipid-rich outer layer

T. Grau*, A. Tschumi, M. Rezwan, M. Dolder, E. C. Böttger & P. Sander

3rd Swiss Molecular Microbiology Workshop (SWIMM), Zurich, Switzerland

The lipoprotein modifying enzyme phospholipid/apolipoprotein transacylase Lnt is found in organisms with a lipid-rich outer layer

T. Grau*, A. Tschumi, M. Rezwan, M. Dolder, E. C. Böttger & P. Sander

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PERSONAL DATA

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EDUCATION & RESEARCH ACTIVITIES

2005-Present:	**Ph.D. thesis** at the Institute of Medical Microbiology, University of Zurich, Switzerland, under supervision of Prof. Dr. Peter Sander and Prof. Dr. Erik C. Böttger. *Lipoprotein Localization Signals and Evaluation of Novel Drug Targets to Combat Tuberculosis*
2002-2003:	Research assistant at the Department of Biochemistry, University of Zurich, Switzerland, in the group of Prof. Dr. Philipp Christen.
2001-2002	**Diploma work/ Master thesis** at the Department of Biochemistry, University of Zurich, Switzerland, under supervision of Dr. Michael F. Koller and Prof. Dr. Philipp Christen. *Induction of Antibodies against Prion Protein with the Help of DnaK*
1996-2002:	**Undergraduate and graduate studies** in biochemistry (**M.Sc.**) at the Faculty of Science (MNF), University of Zurich, Switzerland.
1991-1996	**Secondary school** education (**Matura** Typus E, economics) at the Kantonsschule am Burggraben, St. Gallen, Switzerland.